PATENT COOPERATION T ATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 May 2000 (19.05.00)	
International application No. PCT/US99/22905	**Applicant's or agent's file reference** ISC007/PCT
International filing date (day/month/year) 01 October 1999 (01.10.99)	**Priority date** (day/month/year) 02 October 1998 (02.10.98)
Applicant BAR-OR, David et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 April 2000 (06.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

15

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference ISC.007/PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/22905	International filing date *(day/month/year)* 01 OCTOBER 1999	Priority date *(day/month/year)* 02 OCTOBER 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant ISCHEMIA TECHNOLOGIES, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06 APRIL 2000	08 NOVEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/22905

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-63 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 64-76 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-14 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/22905

III. N n-establishm nt f pini n with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 43-64

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 43-64 .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/22905

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-42	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-42	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-42	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-42 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a method of detecting the occurrence or non-occurrence of an ischemic event in a patient comprising contacting a serum,plasma, fluid, or tissue with metal ions capable of binding to thiol groups in a sample and directly detecting the amount of non-sample bound metal ions by atomic absorption or atomic emission spectroscopy or immunoassay. These methods are specific and sensitive over the prior art of record for detecting several ischemic related events in patients.

---------- NEW CITATIONS ----------
NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): G0IN 21/00, 21/29, 31/22, 33/543, 33/00, 33/53; C12Q 1/00 and US Cl.: 436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
G01N 21/00, 21/29, 31/22, 33/543, 33/00, 33/53, C12Q 1/00

A1

(11) International Publication Number: **WO 00/20840**

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09/165,581	2 October 1998 (02.10.98)	US
09/165,926	2 October 1998 (02.10.98)	US
60/102,738	2 October 1998 (02.10.98)	US
60/115,392	11 January 1999 (11.01.99)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	09/165,581 (CIP)
Filed on	2 October 1998 (02.10.98)
US	09/165,926 (CIP)
Filed on	2 October 1998 (02.10.98)

(71) Applicant *(for all designated States except US)*: ISCHEMIA TECHNOLOGIES, INC. [US/US]; Suite A, 6830 North Broadway, Denver, CO 80221 (US).

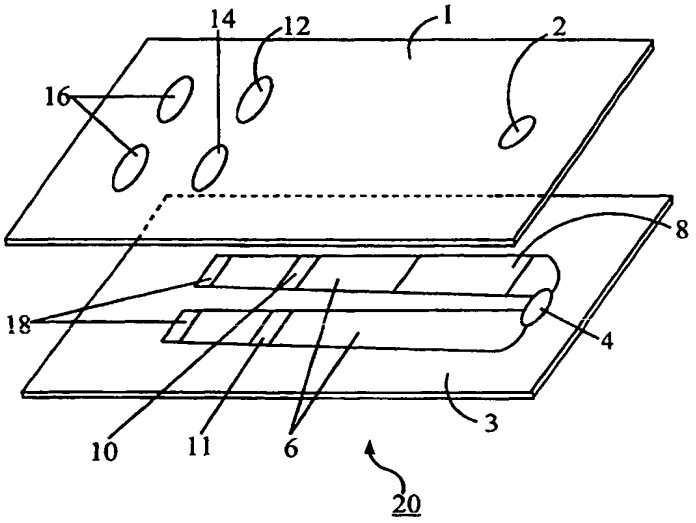
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BAR-OR, David [US/US]; 900 E. Oxford Lane, Englewood, CO 80110 (US). LAU, Edward [US/US]; 4281 Plum Court, Boulder, CO 80301 (US). WINKLER, James, V. [US/US]; 720 Vine Street, Denver, CO 80206 (US).

(74) Agents: SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 East Prentice Avenue, Englewood, CO 80111 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

(54) Title: TESTS FOR THE RAPID EVALUATION OF ISCHEMIC STATES AND KITS



(57) Abstract

The present invention relates to rapid methods for the detection of ischemic states and to kits for use in such methods. Provided for is a rapid method of testing for and quantifying ischemia based upon methods of detecting and quantifying the existence of an alteration of the serum protein albumin which occurs following an ischemic event; methods for detecting and quantifying this alteration include evaluating and quantifying the cobalt binding capacity of circulating albumin, analysis and measurement of the ability of serum albumin to bind exogenous cobalt, detection and measurement of the presence of endogenous copper in a purified albumin sample and use of an immunological assay specific to the altered form of serum albumin which occurs following an ischemic event. Also taught by the present invention is the detection and measurement of an ischemic event by measuring albumin N-terminal derivatives that arise following an ischemic event, including truncated albumin species lacking one to four N-terminal amino acids or albumin with an acetylated N-terminal Asp residue.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G0IN 21/00, 21/29, 31/22, 33/543, 33/00, 33/53; C12Q 1/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, USAPATFUL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,227,307 A (BAR-OR et al) 13 July 1993, see entire document.	1-18 --- 19-35
Y	US 5,290,519 A (BAR-OR et al) 01 March 1994, see entire document.	36-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 DECEMBER 1999	Date of mailing of the international search report 07 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-35 and 36-42

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-35 and 36-42 drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay.

Group II, claims 43-46 and 47-51 drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit.

Group III, claims 52-55 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.1.

Group IV, claims 56 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.2.

Group V, claims 57-64 drawn to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event.

The inventions listed as Groups I to V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay whereas Group II, drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit. These two methods are distinct and different in utilizing different steps, reagents and result in different outcome. Group III is a monoclonal antibody directed to an epitope at the N-terminus of the albumin which lacks the four, three, and two amino acid of SEQ. ID. NO. 1 where as Group IV, drawn to a structurally different monoclonal antibody directed to an epitope at the N-terminus of SEQ. ID. NO. 2. Group V to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event. This method is distinct and different from I and II in utilizing different steps, reagents and result in different outcome.

AMENDED CLAIMS

[received by the International Bureau on 29 February 2000 (29.02.00);
original claims 52-56 and 63 amended; new claims 65-76 added;
remaining claims unchanged (4 pages)]

50. The kit of claim 47, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample with said first solid support, and having an area of immobilized ligand to naturally-occurring albumin and albumin N-terminal derivatives proximate the second end, said second support serving as a control.

51. The kit of claim 50, further comprising and end of process indicator at the second end of said second solid support.

52. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the four N-terminal amino acids of SEQ. ID. NO. 1.

53. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the three N-terminal amino acids of SEQ. ID. NO. 1.

54. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the two N-terminal amino acids of SEQ. ID. NO. 1.

55. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the N-terminal amino acid of SEQ. ID NO. 1.

56. A ligand to an epitope at the N-terminus of SEQ. ID NO. 2.

63. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 9, comprising the steps of:

(a) mixing the calibrator composition solution of claim 57 with a predetermined amount of an excess metal salt, whereby said unbound albumin binds to said excess metal ion, generating unbound metal ions,

(b) contacting the mixture of step (a) with color forming compound to form a colored solution, and

(c) applying the mixture of step (b) to the analyzer, whereby the predetermined ratio of albumin to metal serves as a standard for calibration.

64. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 19, comprising the step of:

applying the calibrator solution of claim 57 wherein the metal is copper to the analyzer to determine the amount of copper ions bound to the albumin N-terminus, whereby the predetermined ratio of albumin to copper serves as a standard for calibration.

65. A method of detecting an albumin N-terminal derivative which lacks four N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 52.

66. A method of detecting an albumin N-terminal derivative which lacks three N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 53.

67. A method of detecting an albumin N-terminal derivative which lacks two N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 54.

68. A method of detecting an albumin N-terminal derivative which lacks an N-terminal amino acid of SEQ. ID NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 55.

69. A method of detecting an albumin N-terminal derivative which is acetylated at its N-terminal Asp residue (SEQ. ID. NO. 2), comprising contacting a sample comprising said derivative with the ligand of claim 56.

70. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an interior filter circle surrounded by an inner concentric ring and an outer concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said inner concentric ring is divided into a first and second half, said first half containing an excess amount of bound metal ion to bind to the N-terminus of said naturally-occurring albumin, and

said outer concentric ring is divided into a first and second half, each said outer ring halves aligned with the inner ring halves, and each said outer ring halves containing ligands to a non-N-terminus epitope of naturally occurring albumin and to albumin N-terminal derivatives.

71. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an inner filter circle surrounded by a concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said concentric ring is divided into a first and second half, said first half having an excess amount of bound metal to bind to the N-terminus of naturally-occurring albumin, and the second half having ligands to a non-N-terminus epitope of naturally-occurring albumin and to albumin N-terminal derivatives.

72. A calibrator composition comprising a predetermined molar ratio of naturally-occurring albumin and albumin N-terminal derivatives, wherein said ratio is between 0.1:1 and 1:0.1.

73. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 3:1.

74. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 1:3.

75. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 1:1.

76. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 9 comprising the steps of:

(a) mixing the calibrator composition of claim 72 with a predetermined amount of an excess metal salt, whereby naturally-occurring albumin binds to said excess metal ion, generating unbound metal ions,

(b) contacting the mixture of step (a) with a color forming compound to form a colored solution,

(c) applying the mixture of step (b) to the analyzer, whereby the predetermined ratio of naturally-occurring albumin to albumin N-terminal derivatives serves as a standard for calibration.

Express Mail No.: EL652339095US

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
G01N 21/00, 21/29, 31/22, 33/543, 33/00, 33/53, C12Q 1/00

A1

(11) International Publication Number: WO 00/20840

(43) International Publication Date: 13 April 2000 (13.04.00)

(21) International Application Number: PCT/US99/22905

(22) International Filing Date: 1 October 1999 (01.10.99)

(30) Priority Data:

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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	09/165,581 (CIP)
Filed on	2 October 1998 (02.10.98)
US	09/165,926 (CIP)
Filed on	2 October 1998 (02.10.98)

(71) Applicant *(for all designated States except US)*: ISCHEMIA TECHNOLOGIES, INC. [US/US]; Suite A, 6830 North Broadway, Denver, CO 80221 (US).

(72) Inventors; and

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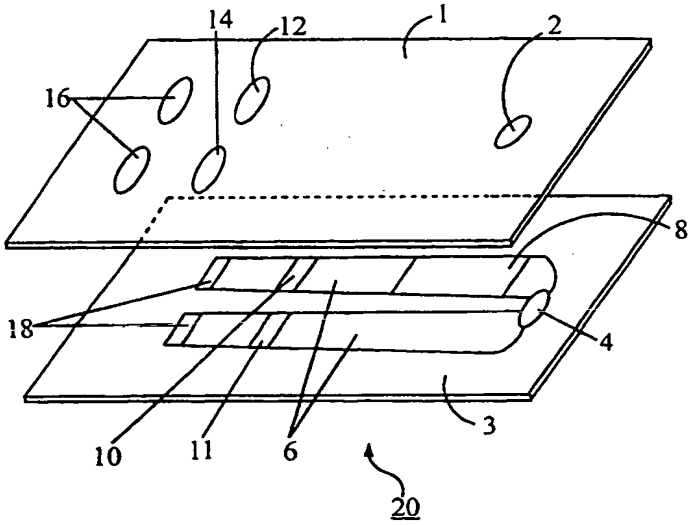

(57) Abstract

The present invention relates to rapid methods for the detection of ischemic states and to kits for use in such methods. Provided for is a rapid method of testing for and quantifying ischemia based upon methods of detecting and quantifying the existence of an alteration of the serum protein albumin which occurs following an ischemic event; methods for detecting and quantifying this alteration include evaluating and quantifying the cobalt binding capacity of circulating albumin, analysis and measurement of the ability of serum albumin to bind exogenous cobalt, detection and measurement of the presence of endogenous copper in a purified albumin sample and use of an immunological assay specific to the altered form of serum albumin which occurs following an ischemic event. Also taught by the present invention is the detection and measurement of an ischemic event by measuring albumin N-terminal derivatives that arise following an ischemic event, including truncated albumin species lacking one to four N-terminal amino acids or albumin with an acetylated N-terminal Asp residue.

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TESTS FOR THE RAPID EVALUATION OF ISCHEMIC STATES AND KITS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to rapid methods for the detection of ischemic states and to kits for use in such methods. More particularly, the invention relates to the measurement of a bound specific transition element to human serum albumin or the measurement of albumin N-terminal derivatives to determine the presence or absence of ischemia.

2. Discussion of the Background

Ischemia is the leading cause of illness and disability in the world. Ischemia is a deficiency of oxygen in a part of the body causing metabolic changes, usually temporary, which can be due to a constriction or an obstruction in the blood vessel supplying that part. The two most common forms of ischemia are cardiovascular and cerebrovascular. Cardiovascular ischemia, in which the body's capacity to provide oxygen to the heart is diminished, is the leading cause of illness and death in the United States. Cerebral ischemia is a precursor to cerebrovascular accident (stroke) which is the third leading cause of death in the United States.

The continuum of ischemic disease includes five conditions: (1) elevated blood levels of cholesterol and other blood lipids; (2) subsequent narrowing of the arteries; (3) reduced blood flow to a body organ (as a result of arterial narrowing); (4) cellular damage to an organ caused by a lack of oxygen; (5) death of organ tissue caused by sustained oxygen deprivation. Stages three through five are collectively referred to as "ischemic disease," while stages one and two are considered its precursors.

Together, cardiovascular and cerebrovascular disease accounted for 954,720 deaths in the U.S. in 1994. Furthermore, more than 20% of the population has some form of cardiovascular disease. In 1998, as many as 1.5 million Americans will have a new or recurrent heart attack, and about 33% of them will die. Additionally, as many as 3 to 4 million Americans suffer from what is referred to as "silent ischemia." This is a condition where no clinical symptoms of ischemic heart disease are present.

There is currently a pressing need for the development and utilization of blood tests able to detect injury to the heart muscle and coronary arteries. Successful

present invention provides specificity and sensitivity levels of 75-95%, which are far more accurate than the EKG exercise stress test and comparable in accuracy to current diagnostic standards. Furthermore, the present invention presents a significant time advantage and is cheaper than competing methods of diagnosis by a factor of at least 15 to 1.

It is known that immediately following an ischemic event, proteins (enzymes) are released into the blood. Well known proteins released after an ischemic heart event include creatine kinase (CK), serum glutamic oxalacetic transaminase (SGOT) and lactic dehydrogenase (LDH). One well known method of evaluating the occurrence of past ischemic heart events is the detection of these proteins in a patient's blood. U.S. Pat. No. 4,492,753 relates to a similar method of assessing the risk of future ischemic heart events. However, injured heart tissue releases proteins to the bloodstream after both ischemic and non-ischemic events. For instance, patients undergoing non-cardiac surgery may experience perioperative ischemia. Electrocardiograms of these patients show ST-segment shifts with an ischemic cause which are highly correlated with the incidence of postoperative adverse cardiac events. However, ST-segment shifts also occur in the absence of ischemia; therefore, electrocardiogram testing does not distinguish ischemic from non-ischemic events. The present invention provides a means for distinguishing perioperative ischemia from ischemia caused by, among other things, myocardial infarctions and progressive coronary artery disease.

method for assessing the patency of an in-situ coronary stent; and, a method for detecting in a pregnant woman the occurrence of placental insufficiency.

Additional advantages, applications, embodiments and variants of the invention are included in the Detailed Description of the Invention and Examples sections.

As used herein, the term "ischemic event," and "ischemic state" mean that the patient has experienced a local and/or temporary ischemia due to partial or total obstruction of the blood circulation to an organ. Additionally, the following abbreviations are utilized herein to refer to the following amino acids:

Amino acid	Three-letter abbreviation	Single-letter notation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

positions 2 and 8 as titrated by $NiCl_2$. Fig. 10A is Peptide 1 at pH 2.55, while 10B is at pH 7.33. Fig. 10C is the spectra at pH 7.30 with 0.3 equiv. $NiCl_2$, and Fig. 10D is pH 7.33 at ~ 1 equiv. $NiCl_2$.

Figs. 11A-D are the ^{1}H-NMR spectra of Peptide 1 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) which shows the methyl signals of the two Ala residues at positions 2 and 8 as titrated by $CoCl_2$. Fig. 11A is Peptide 1 at pH 2.56, while 11B is at pH 7.45. Fig. 11C is the spectra at pH 7.11 with ~0.5 equiv. $CoCl_2$, and Fig. 11D is pH 7.68 at ~ 1 equiv. $CoCl_2$.

Figs. 12A-D are the ^{1}H-NMR spectra of Peptide 1 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) which shows the methyl signals of the two Ala residues at positions 2 and 8 as titrated by $CuSO_4$. Fig. 12A is Peptide 1 at pH 2.56, while 12B is at pH 7.54. Fig. 12C is the spectra at pH 7.24 with ~0.5 equiv. $CuSO_4$, and Fig. 12D is pH 7.27 at ~ 1 equiv. $CuSO_4$.

Figs. 13A-D are the ^{1}H-NMR spectra of Peptide 2, which is the acetylated-Asp version of Peptide 1. Fig. 13A is Peptide 2 at pH 2.63. Fig. 13B is Peptide 2 at pH 7.36. Fig. 13C is Peptide 2 at pH 7.09 with ~0.5 equiv. $NiCl_2$. Fig. 13D is Peptide 2 at pH 7.20 with ~1 equiv. $NiCl_2$.

Figs. 14A-E are the ^{1}H-NMR spectra of Peptide3 (Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys). Fig. 14A is Peptide 3 at pH 2.83. Fig. 14B is Peptide 3 at pH 7.15. Fig. 14C is Peptide 3 at pH 7.28 with ~ 0.13 equiv. $NiCl_2$. Fig. 14D is Peptide 3 at pH 7.80 with ~0.25 equiv. $NiCl_2$. Fig. 14E is Peptide 3 at pH 8.30 with ~0.50 equiv. $NiCl_2$.

Figs. 15A-D are the ^{1}H-NMR spectra of Peptide 4 (His-Lys-Ser-Glu-Val-ala-His-Arg-Phe-Lys). Fig. 15A is Peptide 4 at pH 2.72. Fig. 15B is Peptide 4 at pH 7.30. Fig. 15C is Peptide 4 at pH 8.30 with ~0.5 equiv. $NiCl_2$. Fig. 15D is Peptide 4 at pH 8.10 with ~1 equiv. $NiCl_2$.

Figs. 16A-D are the ^{1}H-NMR spectra of Peptide 5 (Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys). Fig. 16A is Peptide 5 at pH 2.90. Fig. 16B is Peptide 5 at pH 7.19.

"Derivative N-terminus" refers to the 4-12 amino acids at the N-terminus of albumin N-terminal derivatives, which may serve as an epitope in the generation of a monoclonal antibody.

"Endogenous copper" refers to copper present in a patient sample of albumin, i.e., not exogenously added during the diagnostic procedure.

"Excess quantity" of metal ion or "excess metal ion" refers to addition of an amount of metal ion that will substantially exceed the stoichiometrically available albumin metal ion binding sites such that substantially all naturally-occurring albumin is bound to metal ion at its N-terminus.

"Known value" as used herein means a clinically-derived cut-off value or a normal range, to which a measured patient value is compared so as to determine the occurrence or non-occurrence of an ischemic event.

"Naturally-occurring albumin" refers to albumin with an intact N-terminus (Asp-Ala-His-Lys-) that has not been acetylated.

"Purified albumin" or "purified albumin sample" refers to albumin that has been partially purified or purified to homogeneity. "Partially purified" means with increasing preference, at least 70%, 80%, 90% or 95% pure.

"Treadmill test" means a stress test to increase myocardial O_2 demand, while observing if a mismatch occurs between demand and supply by observing symptoms such as shortness of breath, chest pain, EKG, low blood pressure and the like.

While not being bound by any particular theory, it is believed that the present method works by taking advantage of alterations which occur to the albumin molecule, affecting the N-terminus of albumin during an ischemic ("oxygen-depletion") event. (Ischemia occurs when human tissue is deprived of oxygen due to insufficient blood flow.) A combination of two separate phenomena are believed to explain the mechanism by which the ischemia test of the present invention works. First, it is believed that the localized acidosis which occurs during an ischemic event generates free radicals which alter albumin's N-terminus; thus, by detecting and quantifying the existence of altered albumin, ischemia can be detected and quantified.

585 amino acids folded into three homologous domains with one free sulfhydryl group on residue # 34. The specific amino acid content of human albumin is:

Residues:	Asp	Asn	Thr	Ser	Glu	Gln	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Trp	Arg
Number	39	15	30	22	60	23	25	12	63	35	39	6	8	61	18	30	16	58	1	23

In the first embodiment of the present invention, an excess of metal (e.g., cobalt) ions are introduced into a (purified) albumin sample obtained from a patient serum, plasma, fluid or tissue sample (this embodiment is hereafter referred to as the "excess metal embodiment"). In normal (non-ischemic) patients, cobalt will bind to one or more amino acid chains on the N-terminus of albumin. In ischemic patients, however, most likely due to the alteration of the binding site of the N-terminus, cobalt binding to albumin is reduced. Accordingly, the occurrence or non-occurrence of an ischemic state can be detected by the presence and quantity of bound or unbound cobalt. Measurement of cobalt can be conducted by atomic absorption, infrared spectroscopy, high-performance liquid chromatography ("HPLC") or other standard or non-standard methods, including radioactive immunoassay techniques.

The details of the second mechanism are believed to be as follows. Ceruloplasmin is a circulating protein which binds copper; approximately ninety-percent of the in vivo copper (copper is abundant in blood, with concentrations comparable to iron) will be bound to ceruloplasmin. The remainder is in other bound forms; almost no free copper exists in circulating blood. In acidic conditions and reduced oxygen conditions, such as happens during ischemia, ceruloplasmin releases some of its bound copper. The released copper is taken up by albumin. Copper and cobalt both bind to albumin at the same site within the N-terminus. Thus, the bound endogenous copper, present during ischemia, blocks cobalt from binding to albumin. The decrease in cobalt binding capacity of circulating albumin can be measured and quantified as a means for detecting and quantifying the presence of an ischemic event.

The excess metal embodiment of the present invention comprises a method for detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of: (a) contacting a biological sample containing albumin of

in broadening of the resonances associated with the first three residues. Thus, the diamagnetic nature of the nickel complex makes it more amenable for NMR studies.

The excess metal embodiment of the present invention also includes a colorimetric method of detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of: (a) contacting a biological sample containing albumin of said patient with a predetermined excess quantity of a salt of a metal selected from the group consisting of V, As, Co, Cu, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag, to form a mixture containing bound metal ions and unbound metal ions, (b) contacting said mixture with an aqueous color forming compound solution to form a colored solution, wherein said compound is capable of forming color when bound to said unbound metal ion, (c) determining the color intensity of said colored solution to detect the presence of unbound metal ions to provide a measure of bound metal ions, and (d) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event. Preferred embodiments of this method include the additional step of diluting said colored solution with an aqueous solution isosmotic with blood serum or plasma prior to step (c). Also preferred are: using ferrozine as the color forming compound, and, alternatively, using the compound Asp-Ala-His-Lys-R, wherein R is any group capable of forming color when bound to said metal ion as the aqueous color forming compound. Conducting steps (b) and (c) in a pH range of 7 to 9 is preferred. Further, conducting steps (b) and (c) using a spectrophotometer is preferred. Preferred samples in this method include serum, plasma, or purified albumin and a preferred metal ion salt is cobalt.

Another embodiment is based on the endogenous copper mechanism discussed above. This embodiment involves a method for detecting the occurrence or non-occurrence of an ischemic state in a patient comprising the steps of: (a) detecting the amount of endogenous copper ions present in a purified albumin sample of said patient, and (b) correlating the quantity of copper ions present with a known value to determine the occurrence or non-occurrence of an ischemic event. Preferred methods for detection of the amount of copper ions present in the purified albumin sample are

metal ion and albumin is at least 4-5 minutes, and preferably 10 minutes, i.e., an amount of time sufficient for equilibrium to be reached. It is also preferred that heparin be added to the sample prior to the addition of the excess quantity of metal ion.

The partitioning step of the derivative embodiment method can be carried out in two ways. It can be effected by having the excess metal ion of step (a) bound to a solid support such that the resulting albumin-metal complexes are retained on the solid support, permitting the elution separation of the albumin N-terminal derivatives. Alternatively, a solution of excess metal ion can be added to the patient sample, permitting the albumin-metal complexes to form, and the partitioning can be effected by contacting the complexes with antibodies to the metal-albumin complex that are bound to a solid support.

Thus, in one aspect, the derivative embodiment involves a method comprising: (a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of a metal ion bound to a solid support, whereby the metal ion binds to the N-terminus of naturally-occurring albumin, forming metal-albumin complexes; (b) separating the complexes from said derivatives, if any; (c) measuring at least one of said derivatives, if any; and (d) comparing said measured derivative to a known value, whereby the ischemic event may be detected or measured. It is preferred that the solid support of step (a) be a diacetate or a phosphonate matrix. It is also preferred that the metal ion used in step (a) be nickel ion. It is further preferred that copper ion not be used in this method as it is likely to demonstrate non-specific binding to albumin thiol groups (located outside the N-terminus), possibly generating false negative results.

Metal affinity chromatography methods useful in this embodiment are within the skill in the art. For example, resins for separating proteins (including albumin) using metal affinity chromatography are described in U.S. Pat. Nos. 4,569,794; 5,169,936; and 5,656,729.

In another aspect, the derivative embodiment involves a method comprising: (a) contacting a patient sample comprising naturally-occurring albumin and optionally

albumin binds. The albumin N-terminal derivatives continue to migrate down the solid support 6 to an area 10 containing ligand. In preferred embodiments, the ligands at area 10 are antibodies to albumin N-terminal derivatives and/or antibodies to naturally-occurring albumin. An antibody to naturally-occurring albumin may be used at area 10 provided it is directed to an epitope that is not located at the N-terminus of naturally-occurring albumin, so that it may bind to the derivatives. An antibody at area 10 to an albumin N-terminal derivative refers to an antibody directed to an N-terminal epitope of the derivative, such that the antibody is specific (i.e., recognizes only) the particular albumin N-terminal derivative. An advantage of including antibodies to albumin N-terminal derivatives at area 10 is that the amount of each or all N-terminal derivatives can be measured. Measurement of each derivative may permit a more accurate assessment of the degree and timing of the ischemic event. For example, a relatively higher concentration of the derivative lacking four N-terminal amino acids may reflect a greater degree or a longer duration of ischemia than a second sample where another derivative (e.g., albumin lacking only its N-terminal Asp residue) is more prevalent. Although the relative order of appearance of each derivative during the course of an ischemic event has not yet been determined, it will be possible to do so upon correlation of derivatives observed in patient samples with clinical observations of patients from whom the samples have been derived.

In the control (second) elongated solid support 6, an area 11 containing ligand to albumin is provided to detect all albumin, naturally-occurring or N-terminal derivatives, in the sample. Thus, the antibody at area 11 is directed to an albumin epitope that is not located at the N-terminus of albumin. The antibody or antibody mixture at areas 10 and 11 should be the same for control purposes.

The test and control results can be observed through ports 12 and 14, respectively. The binding of albumin or albumin N-terminal derivatives to antibody is detected by methods known in the art such as sandwich assays, enzyme assays or color indicators. For example, a labeled antibody may be added through ports 12 and 14 to bind to any albumin that is bound to antibody attached to areas 10 and 11. The label on the added antibody may be, for example, alkaline phosphatase, a commonly

whereby naturally-occurring albumin-metal complexes have been formed. The circular filter is surrounded by an inner concentric ring divided into a test half 32 which contains ligand (e.g., monoclonal antibody) to albumin-metal complexes, and a control half 34 which contain no ligand. Beyond the inner concentric ring is an outer concentric ring divided into a test half 38 and a control half 36, both of which contain ligand to albumin. In area 36, ligand is provided that detects all albumin, naturally-occurring or N-terminal derivatives, in the sample. Thus, the antibody at area 36 is directed to an albumin epitope that is not located at the N-terminus of naturally-occurring albumin. In area 38, ligand to naturally-occurring albumin and/or to albumin N-terminal derivatives is likewise provided. Again, for control purposes, the antibody or antibody mixture in areas 36 and 38 should be the same.

As the patient sample radiates from the filter 30, the albumin-metal complexes bind to antibody to complexes in area 32. Filtrate from area 32 passes into area 38, where albumin N-terminal derivatives bind to antibody. Likewise, as patient sample radiates through area 34 of the control half and into area 38, all albumin present (naturally-occurring and derivative) binds to antibody present in area 36. The amount of albumin or albumin derivatives bound in area 38 is compared to a known value to determine whether an ischemic event has occurred. The amount of albumin or derivatives in area 38 can also be compared to a scale of known values, such as a color scale, to determine the degree of the ischemic event. The amount of albumin or derivatives bound in area 38 is determined by methods known in the art including sandwich assays, enzyme assays or protein color reagents.

As can be appreciated by those skilled in the art, the embodiment in Figure 2 can also be readily adapted to the derivative embodiment method in which metal ion is bound to the solid support. Specifically, the solid support area 32 would have metal ion bound thereto rather than antibody to albumin-metal complex.

Figure 3 illustrates another kit 60 suitable for the derivative embodiment method employing the solid support bound antibody to albumin-metal complex. The kit 60 comprises a circular solid support 56 with a centrally-located sample application filter 50. The filter 50 is surrounded by a concentric ring which is divided

antibodies to albumin-metal complexes for use in the subject methods either already exist in the art or would be readily obtainable using known methods.

In addition to the foregoing antibodies, the derivative embodiment may also use antibodies to one or more of the albumin N-terminal derivatives. As is set forth in the Examples, it has been found that the albumin derivatives that lack four, three, two and even one N-terminal amino acid have lost the capacity to bind to cobalt. Additionally, full-length albumin that has been acetylated at its Asp residue also cannot bind to cobalt. As is appreciated by the skilled artisan, antibodies that are specific to (i.e., recognize only) each of these derivatives can be obtained using known monoclonal antibody technology. Adjuvants such as KLH may be used to enhance immunogenicity.

Applications, embodiments and methods of the present invention comprising one or more of the aforementioned methods of the present invention include: A method for ruling-out the existence of ischemia in a patient, comprising application of any of the aforementioned methods, including application of any of the subject methods wherein said patient possesses one or more cardiac risk factors, said cardiac risk factors being selected from the group consisting of: age greater than 50, history of smoking, diabetes mellitus, obesity, high blood pressure, high cholesterol, and strong family history of cardiac disease. A variant thereof, comprises subjecting the patient to an exercise treadmill test followed by a second application of the same method, followed by a comparison of the results of the two applications. Comparison of the before and after ischemia diagnostic tests will reveal whether the ischemic event is induced only under the elevated metabolic conditions of exercise. This method may be used to detect the existence of ischemia provoked by exercise in an otherwise asymptomatic patient.

Other embodiments, applications and variants of the present invention include a method for ruling-out the occurrence of an temporally-limited ischemic event in a patient comprising application of any of the subject diagnostic methods; a method of detecting the existence of ischemia in an asymptomatic patient comprising application of any of the subject diagnostic methods; a method for the evaluation of patients

treadmill test, and (d) repeating steps (a) through (c) at additional designated times wherein, results obtained at designated time are compared. This embodiment may be used to evaluate patients with known or suspected ischemic conditions, to assess the patency of an in-situ coronary stent and to assess the efficacy of an angioplasty procedure. Preferred designated time intervals are three months, six months or one year.

The present invention also teaches a method for assessing the efficacy of thrombolytic drug therapy, comprising the application of any of the subject diagnostic methods; and a method for detecting in a pregnant woman the occurrence of placental insufficiency, comprising application of any of the subject diagnostic methods.

The subject invention also includes calibration standards which have known molar ratios of albumin and metal and are useful in calibrating analyzers or kits that employ the subject methods. In one embodiment, the calibrator compositions are standards to be used to generate standard curves for calibration of clinical chemistry analyzers such as the Beckman CX-5™, Roche Cobas Mira™ and Dimension XL™. These analyzers can each detect or measure ischemic events based on the colorimetric version of the excess metal embodiment described herein. The calibrator compositions can also be used to calibrate analyzers such as atomic absorbance or atomic emission spectrophotometers. The calibrator compositions have preselected or predetermined ratios of naturally-occurring albumin and metal ion. In preferred embodiments, the albumin is human, the solution is buffered (e.g., Tris or HEPES), the pH is about 7-8, and the metal is divalent and is selected from the group consisting of cobalt, nickel and copper. Aliquots of these calibrators, under specific conditions, produce a defined absorbance at 470-500 nm, i.e., a standard curve.

The albumin that is used in the calibrators is substantially all naturally-occurring. By "substantially all," it is meant that at least 70%, and with increasing preference, at least 80%, 90% and 95% by weight, of the albumin is naturally-occurring. Without wishing to be bound by theory, it is believed that when the calibrator compositions are placed in solution, the metal ion becomes primarily bound

For quality control purposes, the characteristics of the calibrators can be verified by:

1. measuring their metal to albumin ratio; metal can be measured by atomic absorption, and albumin can be measured by bromo cresol green (BCG) assay;

2. using radioactive Co^{57} albumin binding assay employing a Sepharose column;

3. measuring the absorbance of the calibrators at the appropriate wavelength over time; and

4. measuring the absorbance of mixtures of calibrator solutions and excess cobalt plus coloring reagent, such as dithiothreitol (DTT).

A greater understanding of the present invention and of its many advantages may be had from the following examples, given by way of illustration. The following examples are illustrative of some of the methods, applications, embodiments and variants of the present invention. They are, of course, not to be considered in any way limitative of the invention. Numerous changes and modification can be made with respect to the invention.

EXAMPLE 1

Sample Handling Procedures for Ischemia Testing

The samples which were used in the present invention were obtained from a variety of tissues or fluid samples taken from a patient, or from commercial vendor sources. Appropriate fluid samples included whole blood, venous blood, arterial blood, blood serum, plasma, as well as other body fluids such as amniotic fluid, lymph, cerebrospinal fluid, saliva, etc. The samples were obtained by well known conventional biopsy and fluid sampling techniques. Preferred samples were blood plasma and serum and purified albumin. Purified albumin was isolated from the serum by any of the known techniques, including electrophoresis, ion exchange, affinity chromatography, gel filtration, etc.

Blood samples were taken using Universal Precautions. Peripheral venipuncture was performed with the tourniquet on less than 30 seconds (contralateral

Storage and Delayed Testing Data for the Ischemia Test

		≤ 8 hrs. vs. stat		≤ 24 hr. vs. stat*	
	Plasma	n	20	n	23
	(stored at	% diff	-5.3%	% diff	-4.8%
	room temp)	S.D.	.094	S.D.	.090
	Plasma	n	18	n	40
	(stored at	% diff	1.7%	% diff	1.0%
	4° C)	S.D.	.070	S.D.	.094
	Serum	n	16		
	(stored at	% diff	-12.8%	(not enough	
	room temp)	S.D.	.157	samples)	
	Serum	n	14	n	24
	(stored at	% diff	-7.3%	% diff	-2.7%
	4° C)	S.D.	.040	S.D.	.210

* ≤ 24 hr. test results given here are a total that include the ≤ 8 hr. test sample results.

EXAMPLE 2

Test Method for Detecting Occurrence of Ischemic Event Using Cobalt Binding

The ischemia test (cobalt version) was run as follows: 200 µl of patient sera was added to each of two tubes each containing 50 µl 0.1% $CoCl_2 \bullet 6H_2O$. The mixture was allowed to react at room temperature (18-25° C), or higher, for 5 or more minutes. Thereafter 50 µl 0.01 M dithiothreitol (DTT) was added to one of the two tubes (the "test tube") and 50 µl 0.9% NaCl was added to the second tube (the "background tube"). After two minutes, 1 ml 0.9% NaCl was added to both tubes. A470 spectroscopy measurements were taken of the two tubes. The ischemia test was considered positive if the optical density was greater than or equal to .400 OD (or alternatively a clinically derived cut-off) using a spectrophotometer at OD 470nm.

low density lipoproteins, high cholesterol, and strong family history of cardiac disease), the physician is instructed to order a resting twelve-lead EKG and a chest x-ray. If the twelve-lead EKG shows evidence of an acute myocardial infarction (AMI), the patient is immediately transported to a hospital for intensive cardiac treatment. If the twelve-lead EKG does not show evidence of (AMI), the patient will be scheduled for an outpatient twelve-lead EKG exercise treadmill within the next few days. A blood sample should be drawn immediately before and again after the exercise treadmill test and the ischemia test run on each sample.

If the exercise treadmill test shows definite evidence of cardiac ischemia, usually seen by characteristic changes of the ST segments, dramatic abnormalities of pulse or blood pressure, or anginal chest pain, the patient should be treated for cardiac ischemia and referred to a cardiologist for possible coronary angiogram and angioplasty. If the exercise treadmill test does not show any evidence of cardiac ischemia, or the findings are equivocal, but the ischemia test is abnormal, the patient similarly should be treated for cardiac ischemia and referred to a cardiologist for possible coronary angiogram and angioplasty. (Absent the present invention, such patients with moderate to high cardiac risk factors would be referred to a cardiologist for further (typically invasive) cardiac testing).

If the exercise treadmill test does not show any evidence of ischemic heart disease, or the findings are equivocal, and the ischemia test is normal, the patient may be sent home with no evidence of cardiac ischemia. In comparison, prior to the present invention, in the case where the exercise treadmill test does not show any evidence of cardiac ischemia, or the findings are equivocal, patients with low risk for cardiac ischemia typically would not have any other tests ordered. In such cases, the physician is taking a calculated risk. It is well documented in the medical literature that at least 25 to 55 percent of patients (higher in females) will have some ischemic heart disease which is not found with routine exercise treadmill testing.

tubes to finish the test tubes in 10 second intervals, reagents were added to the "Blank" tubes without timing.

The optical density of each sample set was read using the set's Blank to read absorbance at 470 nm. The cuvette was checked for air bubbles before reading and washed with H_20 between sets. The ischemia test was considered positive if the optical density was greater than or equal to .400 using the spectrophotometer at OD 470 nm.

The results of the ischemia test compared to the diagnosis determined by clinical criteria are as described in the chart below. Four false negatives and three false positives were reported.

Clinical Diagnosis		Ischemia Test	
		+	—
+	99	95	4
—	40	3	37

Study results demonstrated that the ischemia test marker has a higher value in patients with clinically diagnosed ischemia. The diagnostic accuracy of the ischemia test for the chest pain study was above 90 percent (sensitivity, 96.0%; specificity, 92.5%; predictive value, (+)96.9%; predictive value, (-) 90.2%).

EXAMPLE 6

Test Method For Evaluation of Patients Suffering From Chest Pain to Determine the Occurrence or Non-occurrence of a Myocardial Infarction

The following study is proposed to test the ability of the present invention to detect ischemia in the initial hours following the onset of chest discomfort suspicious for cardiac ischemia. The cobalt version of the test is used.

The patient population is limited to male or female persons, 30 years or older, who present to the Emergency Department with complaints of chest discomfort of less than four hours in duration for reasons independent of the study. Patients will be

significant decline in cobalt binding (and an increase in the serum absorbance or unbound metal ion) will occur if tissue ischemia is induced during the exercise thallium treadmill test.

Patients already scheduled for an exercise thallium treadmill test were asked to give their consent for participation which required two tubes of blood (20 cc's) to be drawn up to 5 (five) times before, during and after the exercise thallium treadmill test. Eligible patients consisted of patients who met all of the following criteria: (1) Age: 18 years or older; (2) Male or female; (3) able to provide written informed consent; and (4) referred for exercise thallium treadmill test for reasons independent of this investigation. Patients were excluded from participation in the study if they met any of the following criteria: (1) known concurrent non-cardiac ischemic disease including, but not limited to: transient ischemic attacks, cerebral vascular accident, acute myocardial infarction and intermittent claudication; (2) inability to complete the standard protocol for the exercise portion of the exercise thallium treadmill test; or (3) cardiac arrest during the exercise portion of the exercise thallium treadmill test.

Prior to administration of the exercise thallium treadmill test, a pretreatment evaluation was conducted which included documentation of all current medications, documentation of previous medical history, EKG, laboratory and radiographic test results, and documentation of most recent vital signs and physical examination.

The standard exercise thallium treadmill test procedure was followed at all times. In no instance was the drawing of the additional blood samples for the purpose of the study permitted to subject the patient to additional risk (beyond the drawing of blood), or to in any manner modify the treatment of the patient.

The "standard" exercise thallium treadmill test procedure comprised generally the following: The patient was brought to the exercise test room in a recently fasting state. After initial vital signs and recent history was recorded, the patient was connected to a twelve-lead EKG monitor, an intravenous line was established and the patient was instructed in the use of a treadmill. With the cardiologist in attendance, the patient walked on the treadmill according to the standard Bruce protocol: starting at a slow pace (approx. 1.7 mph) and gradually increasing both the percent grade

Of the 59 patients enrolled (plasma and serum samples tested by the ischemia test method), 11 patients were deleted because of one of the following reasons: a chronically occluded coronary artery and no sample collected later than one hour after exercise, a clinical history of exercise leg pain (claudication), hemolyzed baseline blood samples, patient did not continue with the exercise study or did not agree to further blood tests, patient received an exercycle thallium test instead of a treadmill thallium test and one patient whose chest pain was later determined to be due to pneumonia.

Of the remaining 48 patients, 23 had no history of known ischemic heart disease, 23 had prior ischemic heart disease requiring angioplasty or coronary artery bypass grafts and 2 had prior myocardial infarctions but did not receive angioplasty or coronary artery bypass grafts. In the subgroup of 23 patients with no prior history of ischemic heart disease (using a total outcome score of ≥ 9 and a $\geq 4.7\%$ increase in Ischemia Test values (i.e., absorbance associated with unbound excess metal ion) either one or three hours after exercise as positive for ischemia) there were 2 true positives, 15 true negatives, 6 false positives and 0 false negatives for a sensitivity of 100% and a specificity of 72%.

Using the same criteria for positive exercise thallium treadmill and Ischemia Test results, the entire 48 patients (including patients with and without a prior history of ischemic heart disease) had 6 true positives, 29 true negatives, 11 false positives and 2 false negatives for a sensitivity of 75% and a specificity of 73%.

Changing the positive criteria to a total thallium treadmill outcome score of $\geq$ 10 and a $\geq 5.4\%$ increase in Ischemia Test values one hour after exercise for the entire 48 patients (including patients with and without a prior history of ischemic heart disease) gave 3 true positives, 37 true negatives, 7 false positives and 1 false negative for a sensitivity of 75% and a specificity of 88%.

not possess any of the exclusionary criteria. Patients were excluded if they met any of the following criteria: (1) patients who were to have PTCA performed with a perfusion catheter; (2) patients with known, concurrent ischemic disease including, but not limited to transient ischemic attacks, cerebral vascular accident, acute myocardial infarction and intermittent claudication. Prior to PTCA, a pretreatment evaluation was conducted which included documentation of all concurrent medications and the taking of a blood sample for ischemia test administration and baseline (this occurred after the patient had been heparinized and the sheath placed).

The standard PTCA protocol was followed at all times. In no instance was the drawing of the additional tubes of blood permitted to subject the patient to additional risk (beyond the drawing of the blood), or modify the standard protocol.

The "standard" PTCA protocol generally comprised the following: The patient was transported to the cardiac catheterization laboratory in the fasting state. The right groin draped and prepped in the usual sterile fashion. Local anesthesia was administered consisting of 2% lidocaine injected subcutaneously and the right femoral artery entered using an 18 gauge needle, and an 8 French arterial sheath inserted over a guide wire using the modified Seldinger technique. Heparin, 3000 units, was administered I.V. Left coronary cineangiography was performed using Judkins left 4 and right 4 catheters, and left ventricular cineangiography performed using the automated injection of 30 cc of radiocontrast material in the RAO projection. After review of the coronary angiography, PTCA was performed.

The diagnostic cardiac catheter was then removed from the femoral sheath and exchanged for a PTCA guiding catheter which was then positioned in the right or left coronary ostia. An additional bolus of intravenous heparin, 10,000 units, was administered. A coronary guidewire, usually a 0.014 inch flexible tipped wire, was then advanced across the obstruction and positioned distally in the coronary artery. Over this guidewire, the balloon inflation system was inserted, usually consisting of a "monorail" type balloon dilation catheter. Sequential balloon inflations were made, with angiographic monitoring between inflations. The duration of the inflations

and patients without AMI with significant collateral circulation (NonAMI collateral) -- all of whom were undergoing emergent or elective angioplasty had blood samples collected prior to PTCA, immediately after balloon deflation, 6 hours after the procedure, and 24 hours after the procedure. A total of 63 patients were tested. The standard PTCA protocol (as described in Example 8) was followed.

During PTCA, blood was drawn into a syringe and then transferred to sodium-heparinized tubes. Post PTCA samples were drawn into green top sodium-heparinized tubes. In all other regards, sample collection and administration of the ischemia test occurred essentially as described in Example 5 herein. The test technician was masked to the time the PTCA sample was taken.

The ischemia test was considered positive if it increased between baseline and immediately after balloon angioplasty. The results of the study showed a statistically significant rise ($p=0.0001$) in the ischemia test marker following balloon angioplasty and a return to baseline within 24 hours. The mean percent increase for all patients in the study was 9.4%. .

TIME POINT	N	MEAN	SD	MEAN DIFF FROM BASELINE	SD	MEAN % DIFF FROM BASELINE	SD	P-VALUE
Baseline	62	.354	.0424	.	.	.	.	.
Immed. post PTCA	63	.385	.0411	.0310	.0382	9.4%	.1178	.0001
6 hours post PTCA	57	.368	.0513	.0150	.0505	5.0%	.1507	.0167
24 hours post PTCA	43	.363	.0474	.0090	.0444	3.2%	.1312	.1221

inflated, the stent expands, locks in place and forms a rigid support to hold the artery open.

Stent use has increased significantly in just the past year, and is now used in the vast majority of patients, sometimes as an alternative to coronary artery bypass surgery. A stent may be used as an alternative or in combination with angioplasty. Certain features of the artery blockage make it suitable for using a stent, such as the size of the artery and location of the blockage. It is usually reserved for lesions that do not respond to angioplasty alone due to the reclosure of the expanded artery.

In certain selected patients, stents have been shown to reduce the renarrowing that occurs in 30–40 percent of patients following balloon angioplasty or other procedures using catheters. Stents are also useful to restore normal blood flow and keep an artery open if it has been torn or injured by the balloon catheter.

However, reclosure (referred to as restenosis) is a common problem with the stent procedure. In recent years doctors have used stents covered with drugs that interfere with changes in the blood vessel that encourage reclosure. These new stents have shown some promise for improving the long-term success of this procedure. Additionally, after a stent procedure has been done, patients are often placed on one or more blood thinning agents such as aspirin, Ticlopidine and/or Coumadin in order to prevent or prolong reclosure. Whereas aspirin may be used indefinitely; the other two drugs are used only for four to six weeks.

The present invention provides a mechanism for monitoring the functioning and patency of an *in situ* stent.

Stent patency was tested in the same study and same patient group in which post-myocardial infarction patients were studied (see Example 9). The study results showed significantly lower ischemia test values immediately after and 6 hours after PTCA for those patients with stents. The following data includes patients in the NonAMI subset only. The number of patients varies because investigators were not always able to obtain blood samples at all four draw times.

Other diagnostics techniques typically used are invasive and thus possess greater risk. For instance, transesophageal echocardiography (T.E.E.) is an imaging procedure, in which a tube with a transducer on the end of it is passed down a person's throat and into the esophagus; images from TEE can give very clear pictures of the heart and its structures. Cardiac catheterization is another invasive procedure which allows for measurement and viewing of the pumping ability of the heart muscle, the heart valves and the coronary arteries. The shortcoming of these procedures, however, lies in their invasive nature.

The present invention provides a non-invasive method for diagnosis and measurement of dysrhythmias which can be used in lieu of, or in supplementation of, the aforementioned invasive procedures.

Patients with dysrhythmias undergoing PTCA were predicted to have more ischemia than those without. (Dysrhythmia is cited in the medical literature as a good indicator of ischemia.) In the 63 patient study detailed in Examples 9 and 10, patients were additionally assigned to a dysrhythmia subset if their medical record showed significant dysrhythmia during PTCA. Study results showed significantly higher ischemia test values immediately after and 6 hours after PTCA in patients with significant dysrhythmias. The following data includes patients in all study subsets. The number of patients varies because investigators were not always able to obtain blood samples at all four draw times.

% CHANGE FROM BASELINE	WITH DYSRHYTHMIA			W/O DYSRHYTHMIA			T-TEST
	N	MEAN	SD	N	MEAN	SD	P
Immed Post PTCA	5	.265	.151	57	.079	.103	.0004
6 hrs Post PTCA	5	.204	.175	51	.035	.141	.0150
24 hrs Post PTCA	5	.144	.236	37	.017	.107	.3000

The same procedure was run with a peptide control (no cobalt). The difference in peak size between test (with cobalt) and control (no cobalt) was proportional to the amount of free cobalt and hence ischemia.

The following preliminary experiments illustrate the properties and critical characteristics of the peptide probe.

EXAMPLE 13

Measurement of Cobalt Binding to HSA and Octapeptide using Cold Cobalt Binding Assay

OBJECTIVE: To investigate cobalt binding to the octapeptide and human serum albumin using cold cobalt binding assay.

EXPERIMENTAL: Octapeptide synthesized at the Inorganic Chemistry Department (BAM 1, Pat Ingrey, Cambridge): NH_2-Asp-Ala-His$^+$-Lys$^+$-Ser-Glu-Val-Ala-$CONH_2$

Molecular weight: 855.4 Da.

SOLUTIONS: $CoC1_2$ 0. 1 % (w/v) = 4.2 mM; HSA 3% (w/v)(in 75 mM HEPES pH 7.4) = 0.45 mM; Octapeptide 0.965 mM (in 75 mM HEPES pH 7.4); HEPES 75 mM pH 7.4; DTT 0. 15 % (w/v); NaCl 0.85 % (w/v).

METHOD: Fifty æL 0.1 % $CoCl_2$ was added to tubes each containing 200 æL of 75 mM HEPES pH 7.4 or 0.45 mM HSA in HEPES or 0.965 mM Peptide in HEPES; the tubes were allowed to stand at room temperature for 10 minutes; 50 æL DTT 0.15 % was added to one tube (test tube) and distilled H_20 to the other (control tube); the tubes were maintained for 2 minutes at room temperature; 1 ml NaCl 0.85 % was then added; the absorbance at A470 nm of the test tube versus the blank was measured.

EXAMPLE 15

Spectrophotometric Analysis of the Octapeptide and Octapeptide-Cobalt Complex

OBJECTIVE: It is clear from the previous mass spectrometry evidence that cobalt forms a complex with the octapeptide with a concomitant loss of two possible protons. Metal complexes in general show distinct absorption in the UV range and in many cases these complexes show either a hypochromic or a bathochromic shift in the spectra. These shifts can be correlated to provide the energy of binding. It was therefore anticipated that the octapeptide-cobalt complexation would provide such information.

METHOD: The quartz cuvette contained 800 µl octapeptide + 200 µl H_2O(control) or $CoCl_2$ (complex). Spectra were run from 180 to 800 nm on a single beam spectrophotometer.

CONCLUSIONS: Cobalt and octapeptide individually have peak absorbances at <200 and 225 nm respectively with little overlap. Following addition of a $CoCl_2$ solution to octapeptide (1.1:1) there was no significant shift in the K_{max} (220 nm). The absorption band at this region broadened indicating complex formation, but the result could not be used to determine the binding energy (constant).

EXAMPLE 16

Mass Spectrometry of Octapeptide After the Addition of Cobalt

OBJECTIVE: To investigate whether mass spectral study would provide molecular weight information for the peptide and its corresponding cobalt complex.

METHOD: 20 or 200 æM $CoCl_2$ (100 æl) was added to 22.9 æM octapeptide (100 æl) to give ratios of cobalt: octapeptide of 1 : 1.1 and 8.7 :1 respectively. Mass spectra for the two samples were carried out as per conditions detailed in the previous experiment.

RESULTS: One major molecular ion peak was observed at 855.4 Da representing the octapeptide alone. After the addition of 20 æM cobalt to the octapeptide, two peaks were observed, a major peak at 855.3 representing octapeptide

the second peak representing free octapeptide. Octapeptide-Co^{2+} complex formed in the presence of oxygen gave a higher ratio of complex over free peptide, as indicated by the first peak being the larger of the two. Octapeptide-Co^{2+} complex formed in the absence of oxygen again gave two peaks but the second peak was now the larger of the two, indicating less complex formation.

CONCLUSIONS: It would appear that oxygenated conditions enhance cobalt binding to the octapeptide.

EXAMPLE 18

The Effect of pH on the Octapeptide

OBJECTIVE: To optimize chromatography conditions for analysis of octapeptide by HPLC.

METHOD: The octapeptide was analyzed by HPLC using a KS437 styrene / DVB Polymer column (4.6 mm x 150 mm, pore diameter 100-150 A, 'BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 6.2, 7.5 and 8.0 at a flow rate of 2 ml/min. Peaks were detected at 230 nm.

RESULTS: At pH 6.2, the octapeptide eluted after 1.6 min. At pH 8.0 the retention time had increased to 2.1 min. When the octapeptide was run at pH 7.5, two peaks were observed at 1.6 and 2.1 min.

CONCLUSIONS: The octapeptide exists in two forms depending on pH. The protonated form elutes at pH 6.2, and the deprotonated form at pH 8.0.

EXAMPLE 19

The Effect of pH on the Binding of Cobalt to the Octapeptide

OBJECTIVE: It was reported that the peptide peak 'shifted' when a solution of cobalt chloride was added to the octapeptide. It was decided to investigate this phenomenon fully as this would provide a direct tool for the determination of several parameters of cobalt binding to the octapeptide.

1.25	3	2.3	27	16.8:1
2.25	3	2.3	27	9.3:1
4.5	3	2.3	27	4.7:1
10	3	2.3	27	2.1:1
18	3	2.3	27	1.2:1
36	3	2.3	27	1:1.7
72	3	2.3	27	1:3.4
200	3	2.3	27	1:9.5

HPLC analysis: The octapeptide-cobalt complex was analyzed by HPLC using a KS437 styrene/DVB polymer column (4.6 mm x 150 mm, pore diameter 100-150 A, BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 8.0 at a flow rate of 2 ml/min. Peaks were detected at 230 nm.

RESULTS: Mean % Peak Height:

Final [$CoCl_2$] (mM)	Peak 1 (Octapeptide- Co complex)	Peak 2 (unknown)	Peak 3 (Octapeptide)
0	--	3.72	96.28
0.1	7.44	7.08	85.49
0.125	9.79	7.55	82.66
0.225	15.65	15.66	68.52
0.45	25.36	19.67	54.98
1.0	58.66	--	50.42
1.8	61.19	14.97	23.85
3.6	69.55	13.69	16.76
7.2	71.49	14.47	14.05
20.0	82.17	10.27	7.56

From the table immediately preceding, a plot of Log cobalt concentration versus % peak height for peak 3 was produced using Prism software. The 50 % binding constant as deduced from the exponential graph had a value of 0.6461 mM.

tetrapeptides upon incubation with $CoCl_2$ would allow elucidation of the probable binding site.

METHOD: Octapeptide 1.97 mg / ml (250 æl) was incubated with the endoprotease Lys-C 100 µg/ml (50 æl) at a substrate : enzyme ratio of 100 : 1 (w/w) in 8.3 mM Tricine, 1.6 mM EDTA pH 8.0 at 37° C for 24 h. After digestion, 27 æl of the product was incubated with 200 mM $CoCl_2$ (3 æl) at 20° C for 10 minutes prior to analysis by HPLC. HPLC Analysis: The products from the Lys-C digest were analyzed by HPLC using an amino column (4.6 mm x 250 mm, pore diameter 100 Å, BioDynamics-73) under isocratic conditions of 30 mM Ammonium acetate at pH 8.0 at a flow rate of 1.5 ml / min. Peaks were detected at 230 nm.

RESULTS: When the digested Lys-C products were run on HPLC, two peaks were observed at 2.6 and 8.9 min, designated tetrapeptides 1 and 2 respectively. Similarly after addition of cobalt to the digested products two peaks were again observed. However, tetrapeptide 1 exhibited an increased UV absorption and decreased retention time, eluting at 1.7 min as opposed to 2.6 min.

CONCLUSIONS: The octapeptide was digested at the C terminus of the lysine residue by the endoprotease yielding two tetrapeptides. On addition of cobalt to the endoprotease digested octapeptide, a single tetrapeptide-cobalt complex was formed with tetrapeptide 1. There appeared to be no effect on tetrapeptide 2.

EXAMPLE 23

Mass Spectrometry Analysis of the Tetrapeptide 1-Cobalt Complex

OBJECTIVE: To determine the identity of tetrapeptide 1.

EXPERIMENTAL: Tetrapeptides 1 and 2 were fractionated by HPLC and collected. $CoCl_2$ 1.2 mM (3 æl) was added to tetrapeptide 1 (27 æl) and incubated at room temperature for 10 minutes. Samples were subsequently run on MS as described previously.

Cobalt:Albumin ratio	Solution A, ml	Solution B, ml
0	200	0
0.4	133	67
0.625	100	100
0.83	67	133
1.25	0	200

To make a cobalt:albumin calibrator solution of 2.5:1, 0.94 ml of 0.32M $Co(OAc)_2$ was added to 229 ml of Solution A. This solution was permitted to sit at room temperature for one hour and then stored at 4°C until use.

EXAMPLE 25

Quality Control Characterization of Calibrator Solutions

To obtain a cobalt:albumin ratio, one ml aliquots of each of the five calibrator solutions (each of which had been in storage for 24 hours prior to testing) was placed individually in dialysis bags and dialyzed against 400 ml 50mM Tris-Cl, pH7.2, 0.15M NaCl, with three changes of buffer at room temperature. Three to 5 µl of the dialyzates were withdrawn and analyzed for albumin using 1 ml of the BCG dye from Sigma Chemical Co. Absorbance was read at 628 nm after 30 seconds.

Cobalt was assessed by atomic absorption by Galbraith Laboratories, Inc., Knoxville, Tn.

The cobalt:albumin ratios were found to conform to expected values for all five calibrator solutions.

Added Cobalt, Co:albumin	At equilibrium, Co:albumin
0.4	0.16
0.625	0.26

Calibrator Co:albumin	A470 Day 1	A470 Day 12	A470 Day 20	A470 Day 23
0	0.26	0.26	0.23	0.27
0.4	0.32	0.30	0.28	0.29
0.625	0.33	0.33	0.31	0.31
1.25	0.39	0.40	0.37	0.37
2.5	0.64	0.60	0.60	0.57

Absorbance was plotted against metal concentration originally present in the calibrator solution. The plot was found to be substantially linear over the period studied.

EXAMPLE 27

The NMR Spectra for the Complex of Ni and Albumin N-terminal Amino Acids

Addition of cobalt or nickel chloride to the synthetic albumin N-terminus octapeptide afforded changes in the appearance of the ^{1}H-NMR spectrum for the resonances of the first three amino acid residues, with diagnostic changes of the Ala-2 methyl doublet at 1.35 ppm. Titration with $NiCl_2$ gave a sharp diamagnetic ^{1}H-NMR spectrum, while addition of $CoCl_2$ induced paramagnetism at the binding site resulting in significant broadening to the resonances associated with the three residues bound around the metal sphere. Figure 4 shows selected regions of the ^{1}H-NMR spectra (500 MHz, 10% D_2O in H_2O, 300K) showing the Ala resonances (Ala-2 and Ala-8) of the octapeptide (A) free of any metal, with a Lys-4 methylene resonance appearing between the doublets for Ala2 at about 1.35 ppm and for Ala8 at about 1.4, (B) with 0.5 equiv. of $NiCl_2$ added resulting in a shift of the Ni-bound Ala2 doublet to about 1.3, (C) with 1.0 equiv. of $NiCl_2$ added, (D) with 0.5 equiv. of $CoCl_2$ added, and (e) with 1.0 equiv. of $CoCl_2$ added. In all cases, the appearance and chemical shift of the resonances attributed to Ser-5, Glu-6, Val-7 and Ala-8 did not change significantly

EXAMPLE 29

U.V. Spectroscopic Evidence of Co Binding to Albumin Pep-10

Pep-10 was made into 1 mg/ml solutions and incubated with $CoCl_2$ (0.08%). Spectral scans were obtained (data not shown). There was no apparent difference in the absorbance after addition of cobalt, indicating that Pep-10 does not bind cobalt.

EXAMPLE 30

Copper/Cobalt Competition Binding for Albumin Pep-12

Pep-12 (20 µL of 1 mg/ml or 0.014 µMol) was mixed with 5 µL $CuCl_2$ (0.08% or 0.023 µMol) and 20 µL $CoCl_2$ 0.08% (0.067 µMol). The U.V. spectral curve is shown in Fig. 8A. AcPep-12 (20 µL of 1 mg/ml or 0.014 µMol) was also mixed with 5 µL $CuCl_2$ (0.08% or 0.023 µMol) and 20 µL $CoCl_2$ 0.08% (0.067 µMol). The U.V. spectral curve is shown in Fig. 8B. The $CuCl_2$ was added to Pep-12 and AcPep-12 before addition of $CoCl_2$. No shift or change occurred by this manipulation.

Pep-12 binds copper and cannot therefore display a shift and increase absorbance when cobalt is added. The tails appearing on the peaks in Figs. 8A and 8B are due to absorbance of copper in the U.V. range.

EXAMPLE 31

Enzymatic Acetylation of N-Terminal Pep-8 and Human Serum Albumin

Human serum albumin (Sigma A-1653) was incubated at 37°C for 1 h with N-acetyl transferase and acetyl CoA, and spectral scans were obtained at various times (2-60 minutes). A steady increase at A235 was observed (assuming A235 reflects acetylation), reaching a plateau at about 40 minutes (data not shown).

Likewise, Pep-8 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala), was acetylated according to the following conditions:

	1	2	3	4	5	6	7	8
Pep-8	250 µL	250 µL	250 µL	250 µL				
NAT	50 µL			50 µL		50 µL		50 µL

The addition of $CoCl_2$ also shows binding but the peaks are broader with a shift in the methyl group Ala 2 to 1.7 ppm (Fig. 11). Fig. 11A shows Peptide 1's Ala2 and Ala8 methyl signals at 1.3 (pH 2.56). Fig. 11B shows Peptide 1 at pH 7.45. Fig. 11C shows widening of the 1.3 ppm peak as 0.5 equivalent $CoCl_2$ is added at pH 7.11. Fig. 11D shows a separate peak for Ala2-Me at 1.7 ppm with 1.0 equivalent $CoCl_2$ at pH 7.68. Fig. 11 scans were conducted at 500 MHz, 10% D_20/90% H_2O (Ala-Me region).

The addition of $CuSO_4$ causes even more broadening of both methyl groups at positions 2 and 8 to the point where, after addition of 1 equivalent of $CuSO_4$, both signals are lost (Fig. 12). Fig. 12A shows Peptide 1 at pH 2.56 with Ala2 and Ala8 methyl signals at 1.35 ppm. Fig. 12B shows Peptide 1 at pH 7.54. Fig. 12C shows Peptide 1 with a broadening of the signal at 1.35 ppm, due to about 0.5 equivalent $CuSO_4$ (pH 7.24). Fig. 12D shows Peptide 1 with about 1 equivalent $CuSO_4$ at pH 7.27. Fig. 12 scans were conducted at 500 MHz, 10% D_20/90% H_2O (Ala-Me region).

Peptide 2: The N-Terminal dodecapeptide, Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which the amino group of the N-terminal Asp has been acetylated.

Addition of $NiCl_2$ to the acetylated derivative does not result in binding, i.e., there is no appearance of additional peaks (Fig. 13). However, addition of even one equivalent of $NiCl_2$ broadens the spectrum considerably due to the fact that the nickel is free in solution. Fig. 13A shows Peptide 2 at pH 2.63 with the Ala2 and Ala8 Me signals at about 1.28 ppm. Fig. 13B shows Peptide 2 at pH 7.36. Fig. 13C shows Peptide 2 with about 0.5 equivalent $NiCl_2$ at pH 7.09. Fig. 13D shows Peptide 2 with about 1 equivalent $NiCl_2$ at pH 7.20. Fig. 13 scans were conducted at 800 MHz, 10% D_20/90% H_20 (Ala-Me region).

Peptide 3: The N-Terminal Unodecapeptide, Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which the terminal Asp is missing.

The addition of $NiCl_2$ (Fig. 17), $CoCl_2$ (Fig. 18) and $CuSO_4$ (Fig. 19) all gave diagnostic changes consistent with metal ion binding. The spectra resemble those obtained with the dodecapeptide (Peptide 1) and not those obtained with Peptides 2, 3, 4 and 5.

Fig. 17A is the N-terminal tetrapeptide at pH 2.49 with an Ala2 signal at 1.3 ppm. Fig. 17B is the tetrapeptide at pH 7.44. Fig. 17C is the tetrapeptide with about 0.8 equivalent $NiCl_2$ at pH 7.42. Fig. 17D is the tetrapeptide with about 1 equivalent $NiCl_2$ at pH 7.80.

Fig. 18A is the tetrapeptide at pH 7.44 with the Ala2 peak at 1.3 ppm. Fig. 18B is the tetrapeptide with about 0.3 equivalent $CoCl_2$ at pH 7.23. Fig. 18C is the tetrapeptide with about 0.8 equivalent $CoCl_2$ at pH 7.33.

Fig. 19A is the tetrapeptide at pH 7.31 with the Ala2 signal at 1.3 ppm. Fig. 19B is the tetrapeptide with about 0.5 equivalent $CuSO_4$ at pH 7.26. Fig. 19C is the tetrapeptide with about 1.0 equivalent $CuSO_4$ at pH 7.32.

Figs. 17-19 scans were conducted at 800 MHz, 10% H_20/90% D_2O (Ala-Me region).

* * * * *

The above description of the invention is intended to be illustrative and not limiting. Various changes or modification in the embodiments described may occur to those skilled in the art. These can be made without departing from the spirit or scope of the invention.

8. The method of claim 6, wherein said immunological assay is conducted using an antibody to a human serum albumin-metal complex.

9. A method of detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of:

(a) contacting a biological sample containing albumin from said patient with a predetermined excess quantity of a salt of a metal selected from the group consisting of V, As, Co, Cu, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag, to form a mixture containing metal ions bound to the N-terminus of albumin and unbound metal ions,

(b) contacting said mixture with an aqueous color forming compound solution to form a colored solution, wherein said compound forms color when bound to said unbound metal ion,

(c) determining the color intensity of said colored solution to detect the presence of unbound metal ions to provide a measure of bound metal ions, and

(d) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event.

10. The method of claim 9, wherein said aqueous color forming compound comprises the compound Asp-Ala-His-Lys-R, wherein R is any group capable of forming color when bound to said metal ion.

11. The method of claim 9, wherein said sample is serum or plasma.

12. The method of claim 9, wherein said sample is purified albumin.

13. The method of claim 9, wherein said metal ion salt is a salt of cobalt.

17. A method for comparing levels of ischemia in patients at rest and during exercise, comprising application of the following steps at designated times:

(a) application of the method of claim 1 or 9 at a first designated time,

(b) administration of an exercise treadmill test followed by a second application of the same method employed in step (a),

(c) comparing the results of step (a) with the results obtained in step (b), and

(d) repeating steps (a) and (b) at additional designated times,

wherein results obtained designated at each designated time are compared.

18. The method of claim 17, wherein said designated times are three months, six months and one year.

19. A method for detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of:

(a) detecting the amount of endogenous copper ions present in a purified albumin sample from said patient, and

(b) correlating the quantity of copper ions present with a known value to determine the occurrence or non-occurrence of an ischemic event.

20. The method of claim 19, wherein said detecting step is conducted using atomic absorption spectroscopy, atomic emission spectroscopy or an immunological assay.

21. The method of claim 20, wherein said immunological assay is conducted using an antibody specific to an antigen comprising the compound Asp-Ala-His-Lys-R, wherein R is copper.

22. The method of claim 20, wherein said immunological assay is conducted using an antibody to a human serum albumin-copper complex.

26. A method for comparing levels of ischemia in patients at rest and during exercise, comprising application of the following steps at designated times:

(a) application of the method of claim 19 at a first designated time,

(b) administration of an exercise treadmill test followed by a second application of the method of claim 19,

(c) comparing the results of the application of the method of claim 19 prior to administration of the exercise treadmill test with the results of the application of the method of claim 19 after administration of the exercise treadmill test, and

(d) repeating steps (a) and (b) at additional designated times,

wherein results obtained at said designated times are compared.

27. The method of claim 26, wherein said designated times are three months, six months and one year.

28. A method of detecting or measuring an ischemic event in a patient comprising:

(a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of metal ion that binds to the N-terminus of naturally-occurring albumin, whereby albumin-metal complexes are formed,

(b) partitioning the complexes from said derivatives, if any,

(c) measuring at least one of said derivatives, if any, and

(d) comparing said measured derivative to a known value, whereby the ischemic event may be detected or measured.

29. The method of claim 28 wherein said metal is Ni or Co.

30. The method of claim 28 wherein said metal of step (a) is bound to a solid support and said partitioning step (b) comprises separating said derivatives from the solid support to which the metal is bound.

36. An immunoassay diagnostic kit for an ischemic event comprising:

an excess quantity of a metal ion to mix with a patient sample which comprises naturally-occurring albumin and optionally albumin N-terminal derivatives, said naturally-occurring albumin forming a complex with said metal ion,

a first elongated solid support having a first and a second end, said first end having a filter for application of said patient sample mixture, an area of immobilized antibody to said albumin-metal complex between the first end the second end, and an area of immobilized ligand to albumin proximate the second end,

whereby after application of said mixture of patient sample and metal ion to said filter, said albumin-metal complex is immobilized at said area of immobilized antibody, and said albumin N-terminal derivatives migrate and bind to the albumin ligand proximate the second end.

37. The kit of claim 36, wherein said metal ion is cobalt ion.

38. The kit of claim 36, further comprising an end of process indicator at the second end of said solid support.

39. The kit of claim 36, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample mixture with said first elongated support, and having an area of immobilized ligand to albumin between the first and second ends, said second support serving as a control.

40. The kit of claim 39, further comprising an end of process indicator at the second end of said second solid support.

43. A method of detecting or measuring an ischemic event in a patient comprising:

(a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of a metal ion bound to a solid support, whereby the metal ion binds to the N-terminus of naturally-occurring albumin, forming albumin-metal complexes,

(b) separating the complexes from said derivatives, if any,

(c) measuring at least one of said derivatives, if any, and

(d) comparing said measured derivative to known value, whereby the ischemic event may be detected or measured.

44. The method of claim 43 wherein the metal ion is nickel ion.

45. The method of claim 43 wherein the solid support is a diacetate or a phosphonate matrix.

46. The method of claim 43 wherein said measuring step (c) comprises contacting said derivative with an antibody to the derivative.

47. A metal affinity diagnostic kit for an ischemic event comprising:

a first elongated solid support having a first and a second end, said first end having a filter for application of a patient sample, an area of immobilized metal ion between the first and the second end, and an area of immobilized ligand to naturally-occurring albumin or albumin N-terminal derivatives proximate the second end.

48. The kit of claim 47, wherein said immobilized metal is nickel.

49. The kit of claim 47, further comprising an end of process indicator at the second end of said first solid support.

57. A calibrator composition comprising a predetermined molar ratio of naturally-occurring albumin and a metal that complexes to the N-terminus of said albumin, whereby complexed albumin and unbound albumin form when said composition is in aqueous solution, wherein said ratio is between 0.1:1 and 1:0.1.

5

58. The composition of claim 57 wherein said metal is selected from the group consisting of Cu, Ni and Co.

59. The composition of claim 57 wherein the predetermined ratio of albumin to
10 metal is 3:1.

60. The composition of claim 57 wherein the predetermined ratio of albumin to metal is 1:3.

15 61. The composition of claim 57 wherein the predetermined ratio of albumin to metal is 1:1.

62. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 1, comprising the step of:

20 applying the calibrator solution of claim 57 to the analyzer to determine the amount of metal ions bound to the albumin N-terminus, whereby the predetermined ratio of albumin to metal serves as a standard for calibration.

25

AMENDED CLAIMS

[received by the International Bureau on 29 February 2000 (29.02.00);
original claims 52-56 and 63 amended; new claims 65-76 added;
remaining claims unchanged (4 pages)]

50. The kit of claim 47, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample with said first solid support, and having an area of immobilized ligand to naturally-occurring albumin and albumin N-terminal derivatives proximate the second end, said second support serving as a control.

51. The kit of claim 50, further comprising and end of process indicator at the second end of said second solid support.

52. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the four N-terminal amino acids of SEQ. ID. NO. 1.

53. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the three N-terminal amino acids of SEQ. ID. NO. 1.

54. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the two N-terminal amino acids of SEQ. ID. NO. 1.

55. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the N-terminal amino acid of SEQ. ID NO. 1.

56. A ligand to an epitope at the N-terminus of SEQ. ID NO. 2.

69. A method of detecting an albumin N-terminal derivative which is acetylated at its N-terminal Asp residue (SEQ. ID. NO. 2), comprising contacting a sample comprising said derivative with the ligand of claim 56.

70. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an interior filter circle surrounded by an inner concentric ring and an outer concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said inner concentric ring is divided into a first and second half, said first half containing an excess amount of bound metal ion to bind to the N-terminus of said naturally-occurring albumin, and

said outer concentric ring is divided into a first and second half, each said outer ring halves aligned with the inner ring halves, and each said outer ring halves containing ligands to a non-N-terminus epitope of naturally occurring albumin and to albumin N-terminal derivatives.

71. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an inner filter circle surrounded by a concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said concentric ring is divided into a first and second half, said first half having an excess amount of bound metal to bind to the N-terminus of naturally-occurring albumin, and the second half having ligands to a non-N-terminus epitope of naturally-occurring albumin and to albumin N-terminal derivatives.

72. A calibrator composition comprising a predetermined molar ratio of naturally-occurring albumin and albumin N-terminal derivatives, wherein said ratio is between 0.1:1 and 1:0.1.

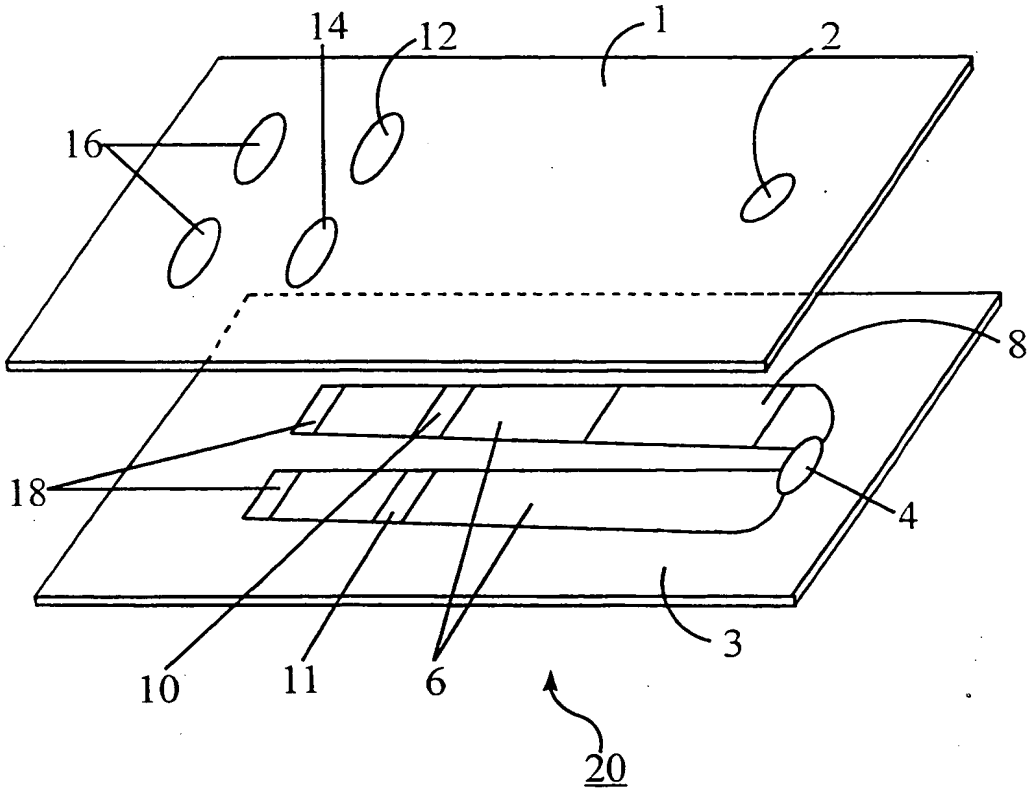


Fig. 1

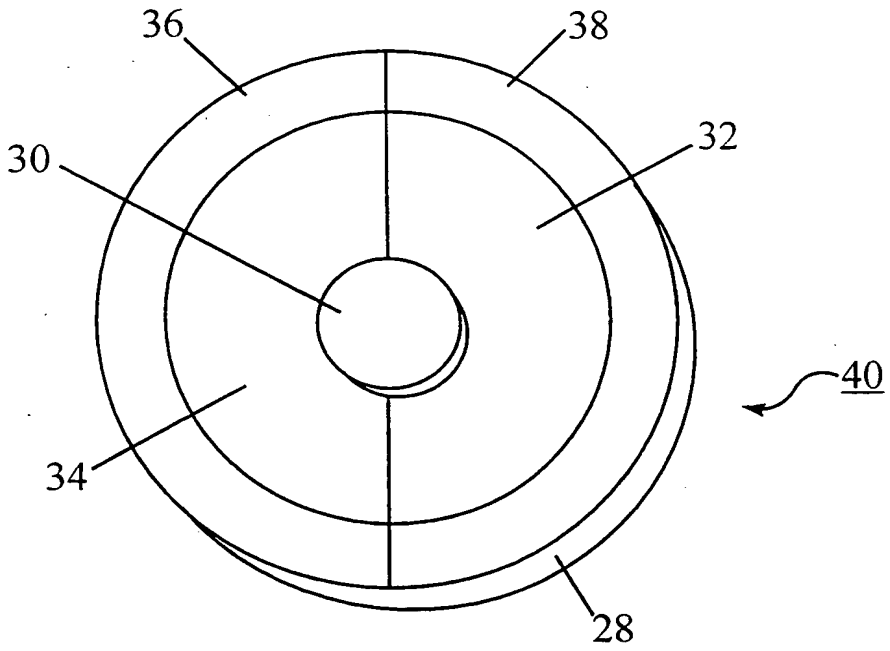
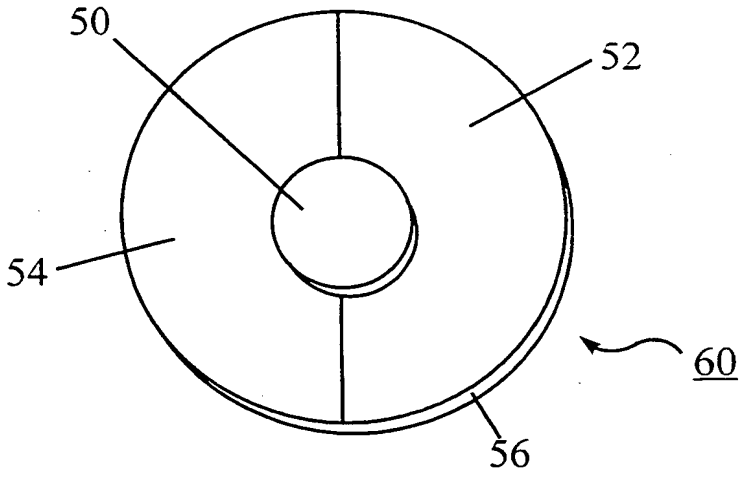


Fig. 2



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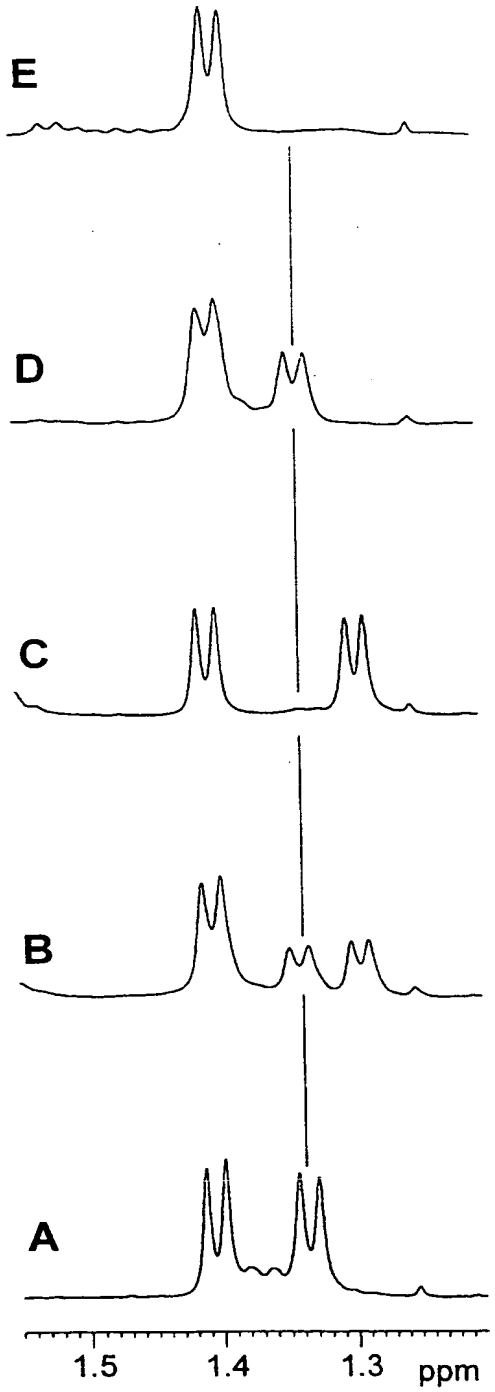

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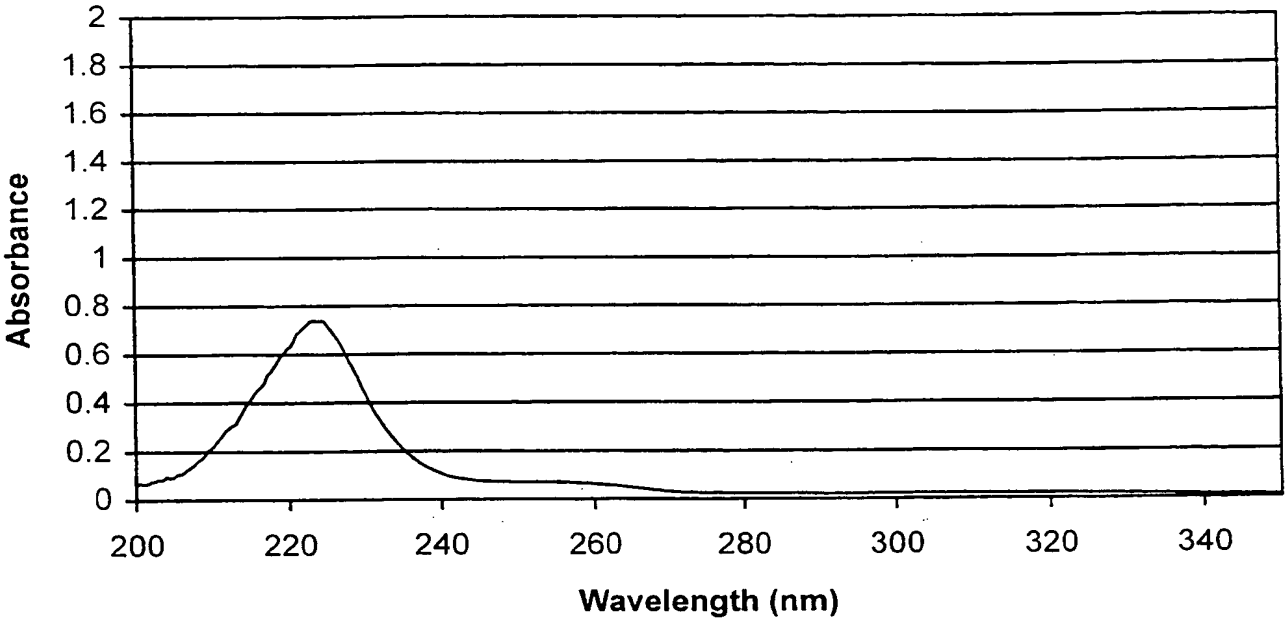

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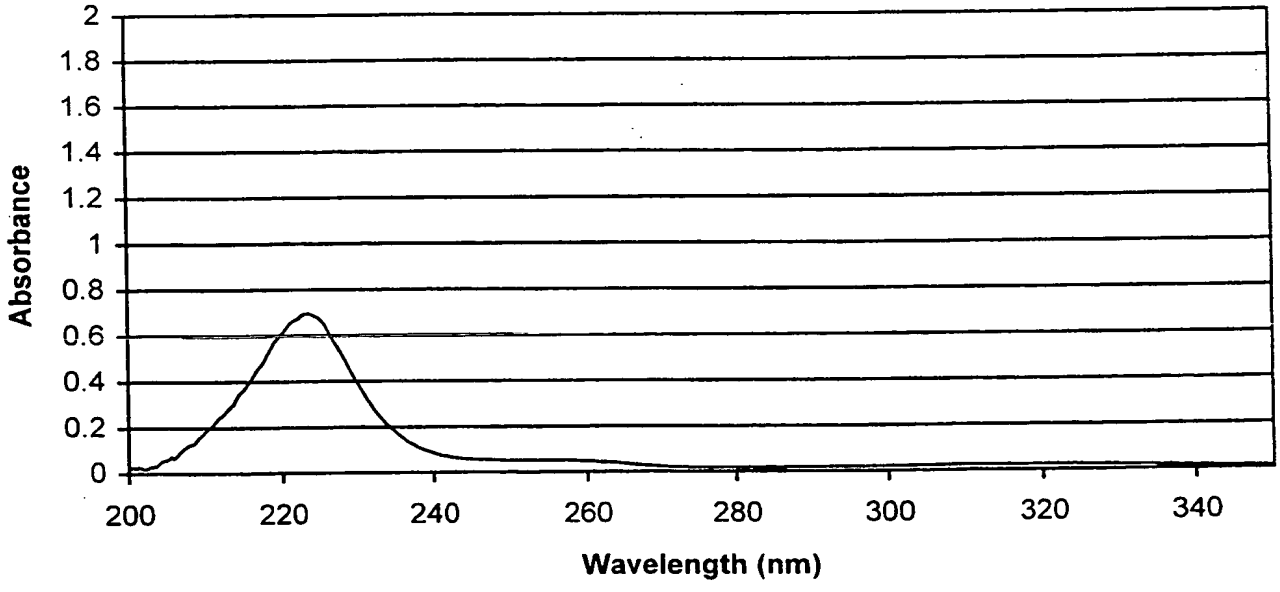

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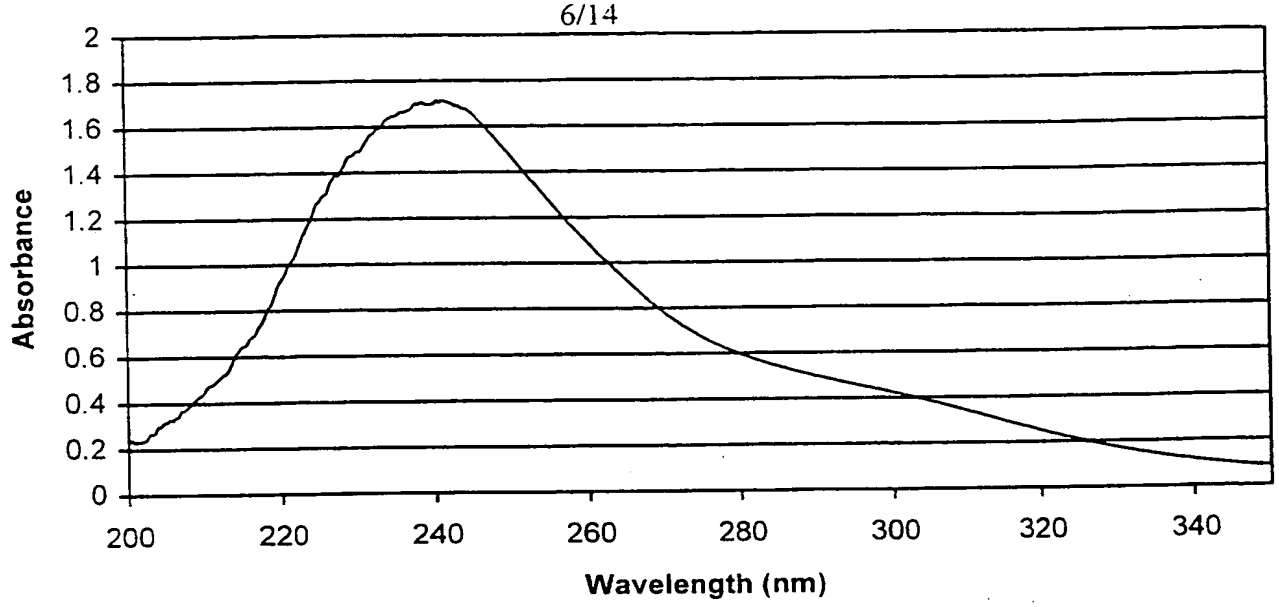

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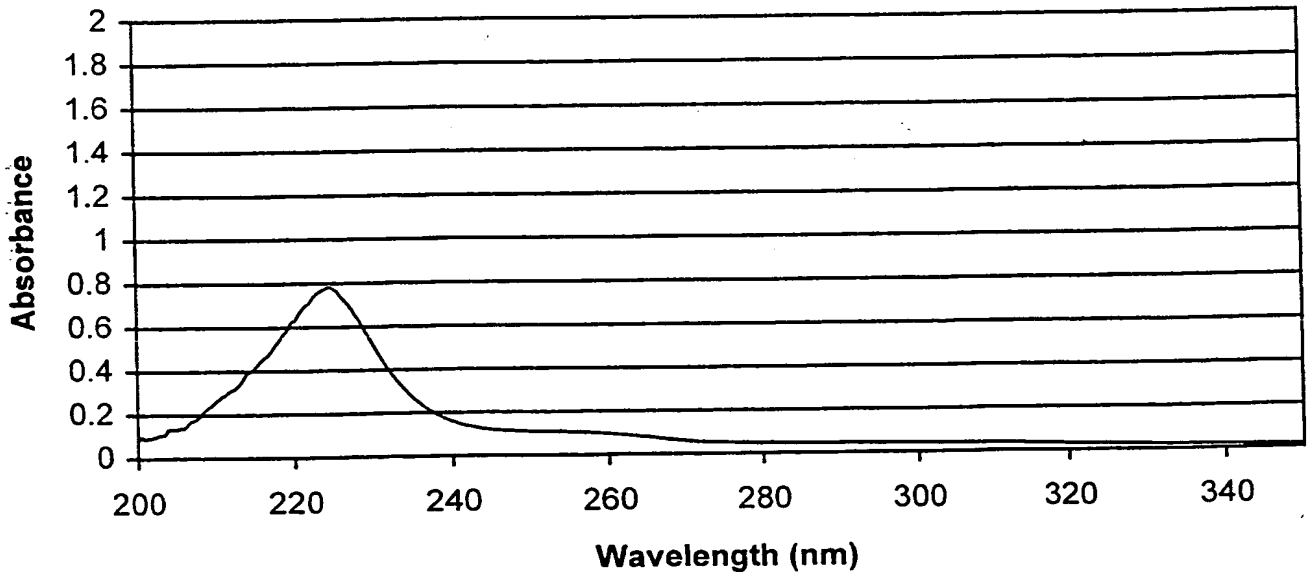

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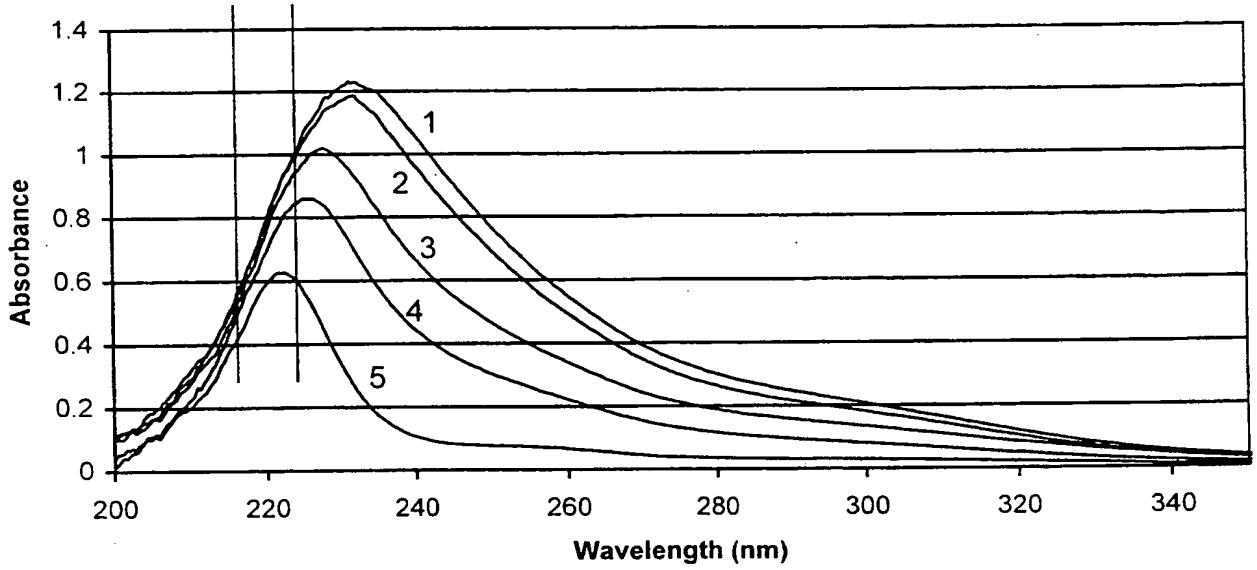
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5

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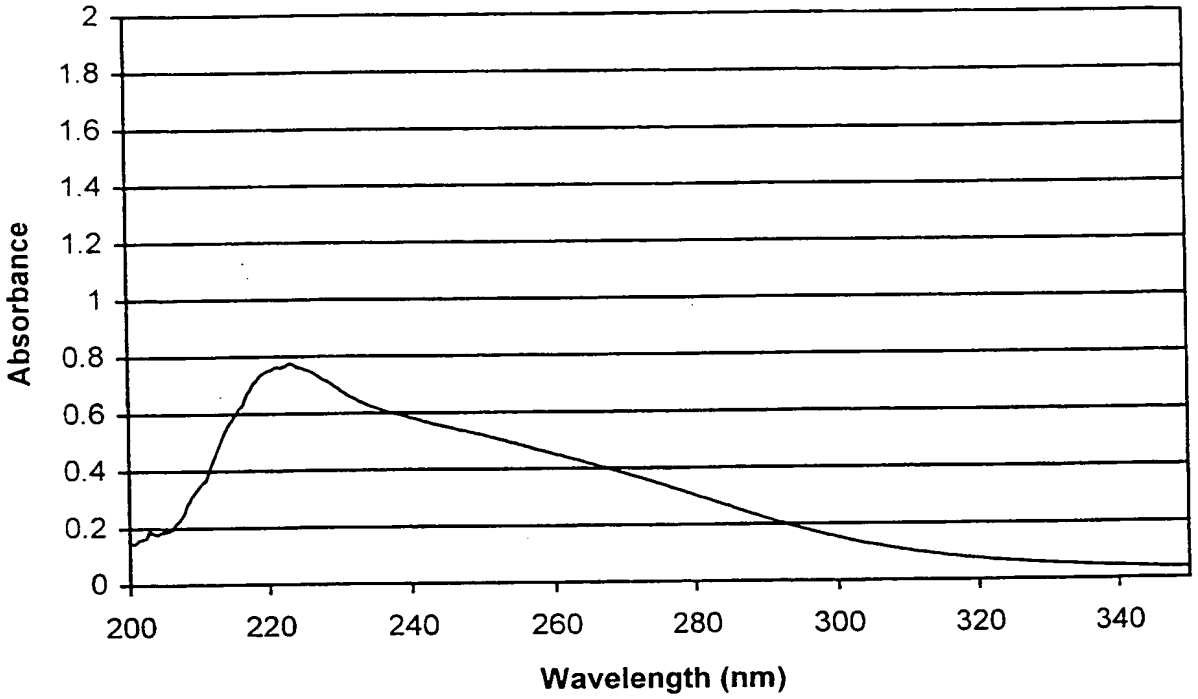

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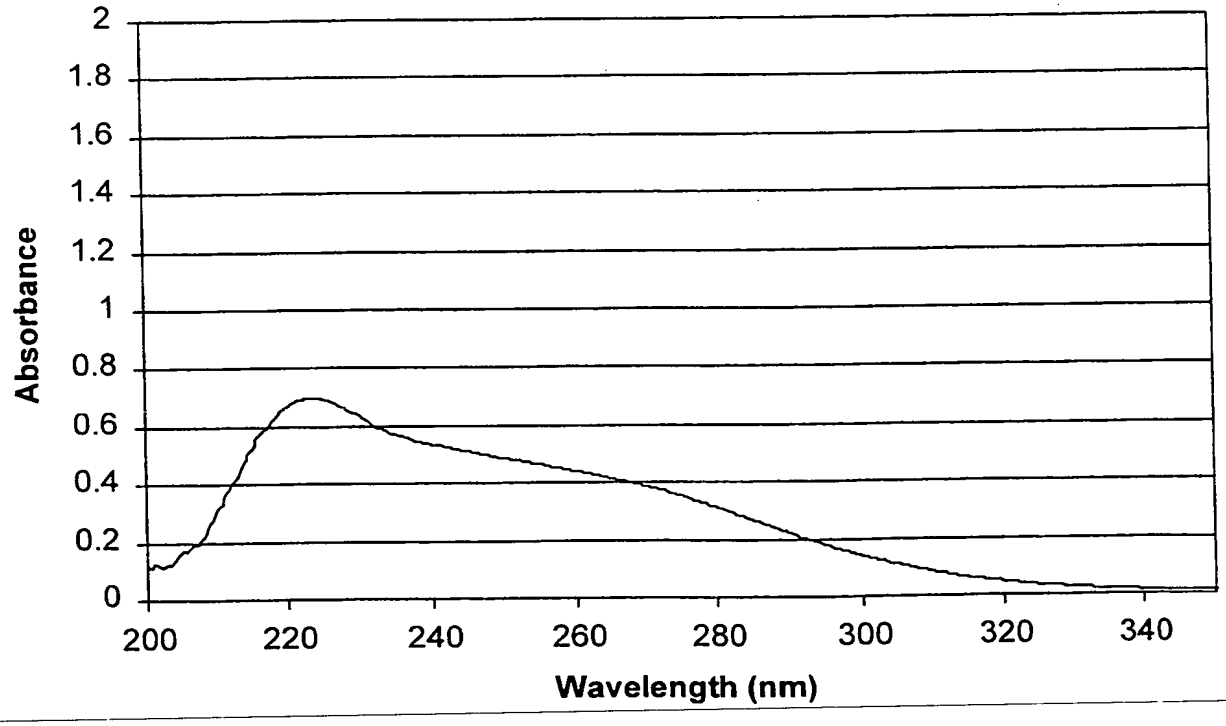

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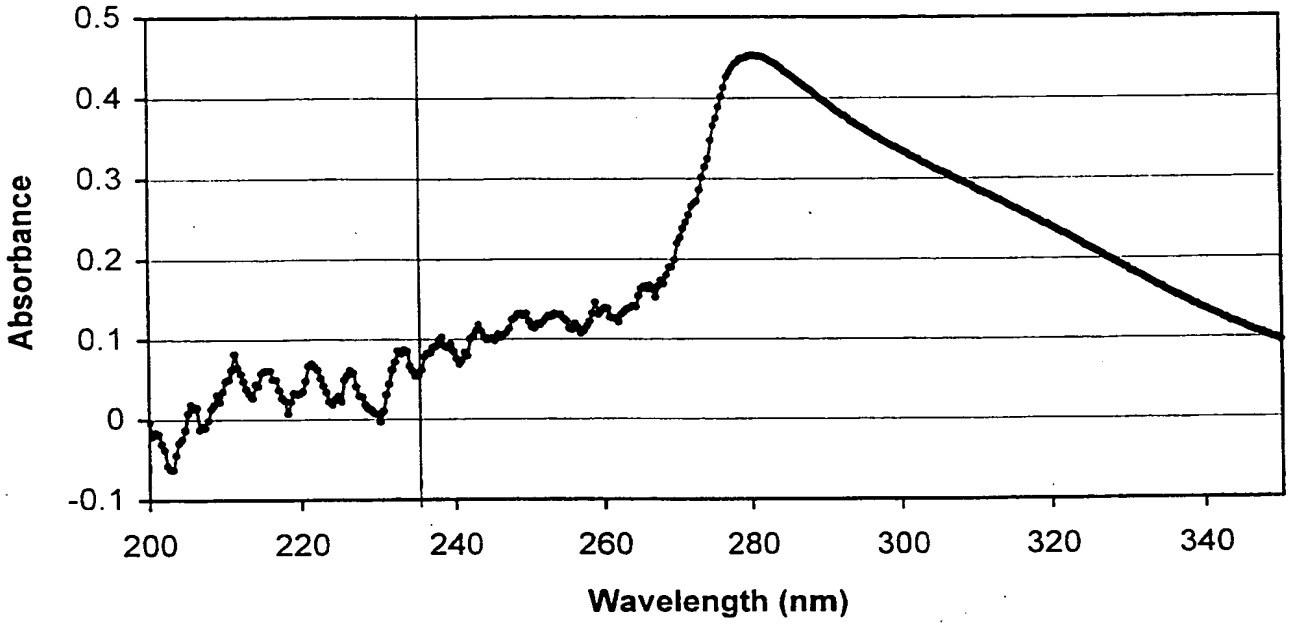
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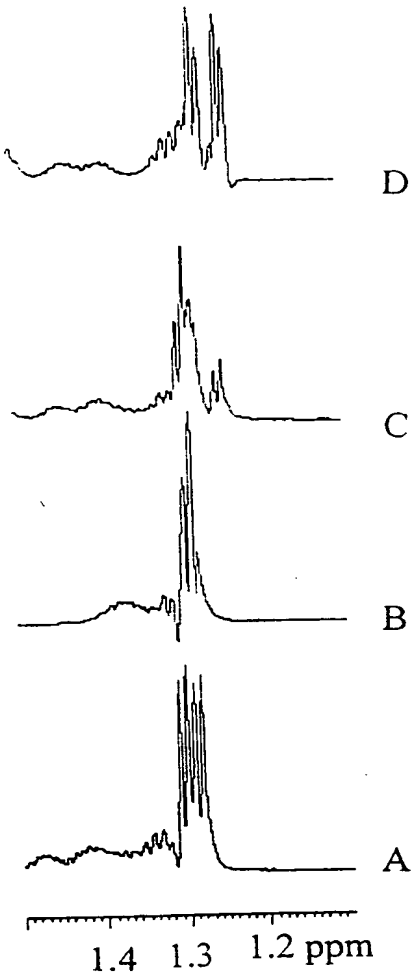
Wavelength (nm)

Figure 9



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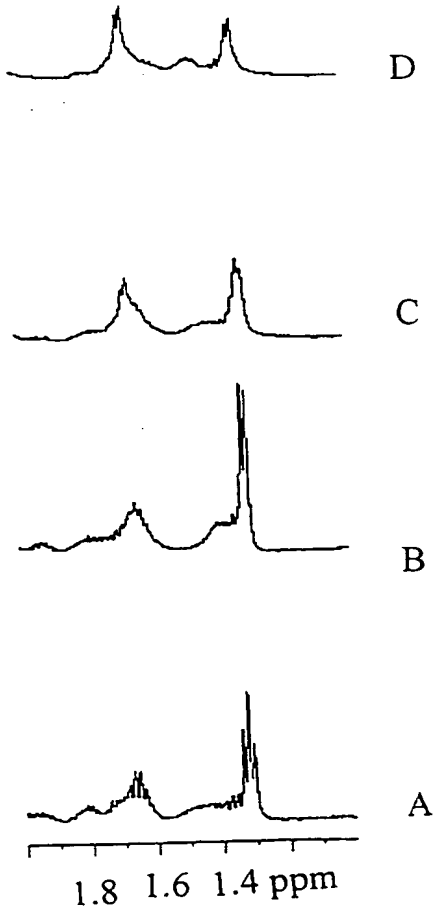

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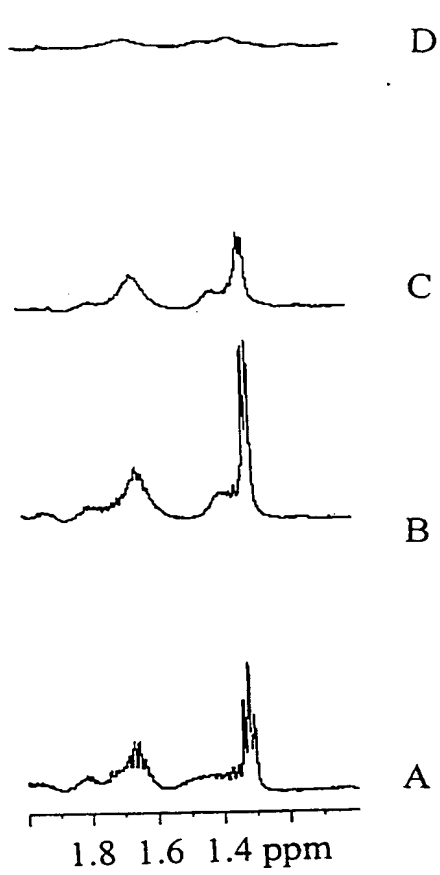

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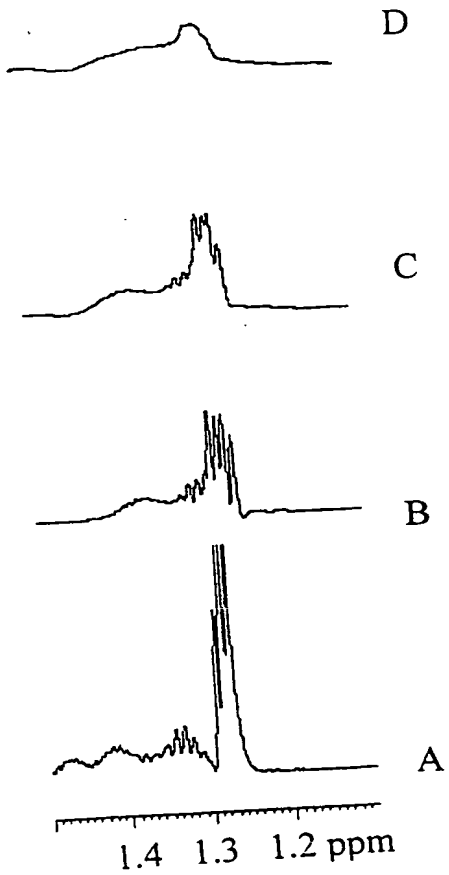

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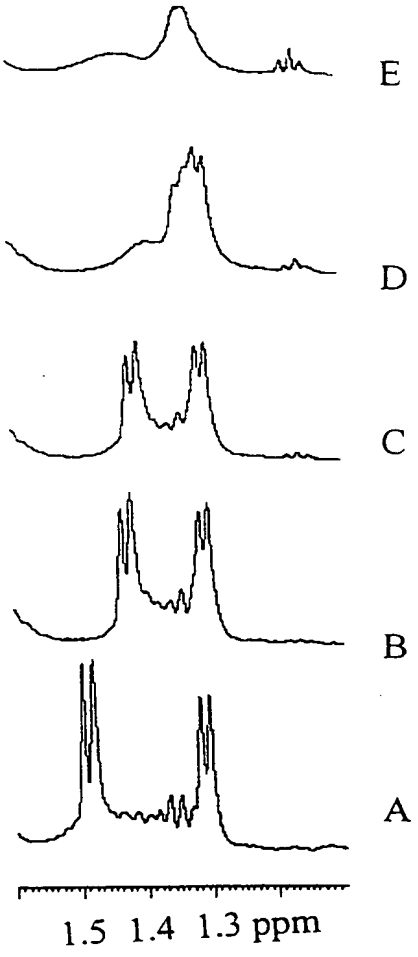

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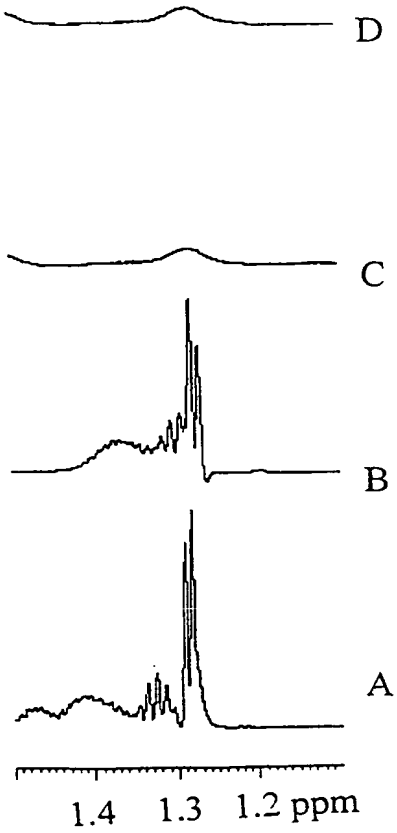

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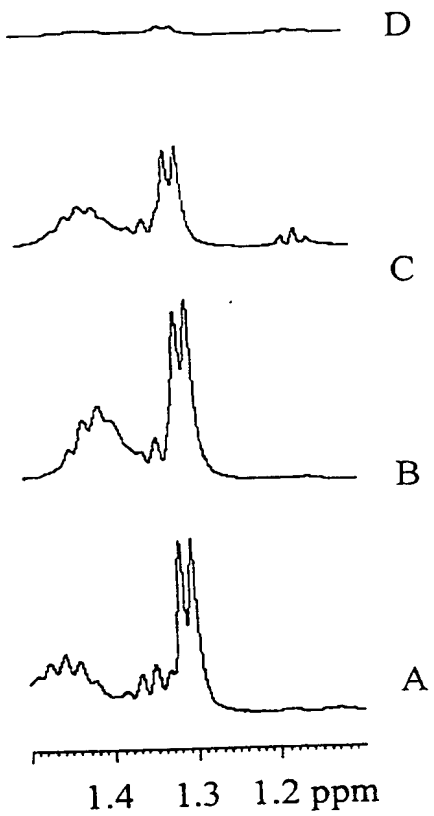

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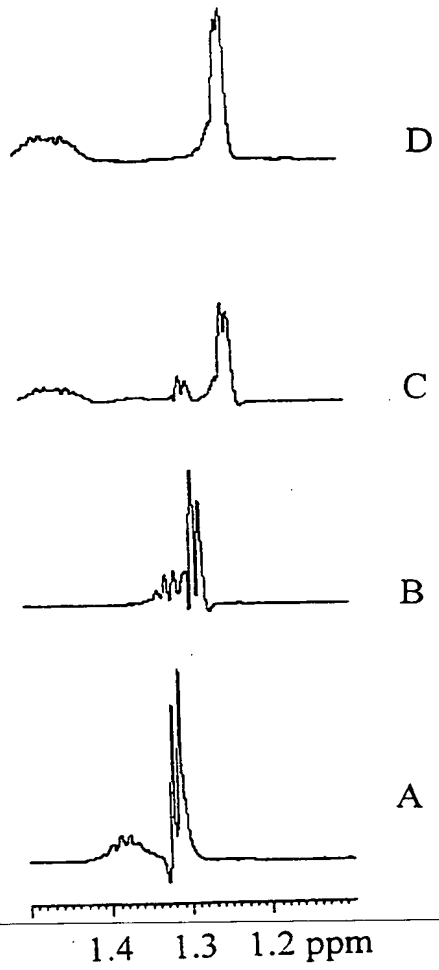

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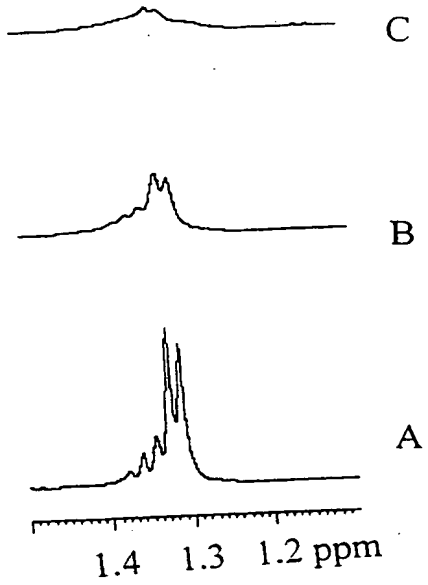

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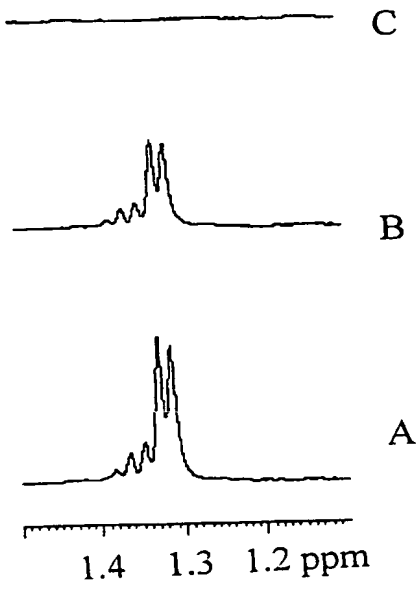

Figure 19

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 21/00, 21/29, 31/22, 33/543, 33/00, 33/53; C12Q 1/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, USAPATFUL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,227,307 A (BAR-OR et al) 13 July 1993, see entire document.	1-18 --- 19-35
Y	US 5,290,519 A (BAR-OR et al) 01 March 1994, see entire document.	36-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 DECEMBER 1999	Date of mailing of the international search report 07 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-35 and 36-42 drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay.

Group II, claims 43-46 and 47-51 drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit.

Group III, claims 52-55 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.1.

Group IV, claims 56 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.2.

Group V, claims 57-64 drawn to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event.

The inventions listed as Groups I to V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay whereas Group II, drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit. These two methods are distinct and different in utilizing different steps, reagents and result in different outcome. Group III is a monoclonal antibody directed to an epitope at the N-terminus of the albumin which lacks the four, three, and two amino acid of SEQ. ID. NO. 1 where as Group IV, drawn to a structurally different monoclonal antibody directed to an epitope at the N-terminus of SEQ. ID. NO. 2. Group V to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event. This method is distinct and different from I and II in utilizing different steps, reagents and result in different outcome.